The role of systemically perturbed PTEN and PKBβ/AKT2 signaling in accumulation of hepatic lipids

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A. List of abbreviations

Less frequently used abbreviation are defined upon their first use in the text.

ACC	acetyl-CoA carboxylase
AMP	adenosine monophosphate
APOB	apolipoprotein B
ATP	adenosine triphosphate
BMI	Body Mass Index
DAG	diacylglycerol
ERK	extracellular signal-regulated kinase
FAS	fatty acid synthase
FoxO1	forkhead box O1
G6Pase	glucose-6-phosphatase
GSK3β	glycogen synthase kinase 3β
GTP	guanosine-5'-triphosphate
IKKβ	inhibitor of nuclear factor κB kinase β
INSR	insulin receptor
IRS1/2	insulin receptor substrate 1/2
JNK	c-Jun N-terminal protein kinase
MAPK	mitogen-activated protein kinase
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NAFLD	non-alcoholic fatty liver disease

PEPCK	phosphoenolpyruvate carboxykinase 1
PGC1α	peroxisome proliferator-activated receptor gamma, coactivator 1α
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinases
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphat
Pkbβ$^{-/-}$	*Pkbβ*-deficiency
PKB	protein kinase B/AKT
PKC	protein kinase C
PP2A	protein phosphatase 2A
PPARγ	peroxisome proliferator-activated receptor g
PTEN	phosphatase and tensin homolog
Pten$^{+/-}$	*Pten*-haplodeficiency
PTP1b	protein tyrosine phosphatase, non-receptor type 1
Raptor	regulatory associated protein of MTOR, complex 1
Rheb	Ras homolog enriched in brain
Rictor	RAPTOR independent companion of MTOR, complex 2
S6K	ribosomal S6 kinase
SREBP1-c	sterol regulatory element binding transcription factor 1
T2D	type 2 diabetes mellitus
TSC1/2	tuberous sclerosis complex 1/2

B. Summary

Non-alcoholic fatty liver disease (NAFLD) is a major health problem and occurs frequently in the context of obesity and type 2 diabetes mellitus (T2D). Insulin resistance of the liver and/or peripheral tissues is considered to drive ectopic lipid accumulation in hepatocytes, but individual contributions are not fully understood. Hepatocyte-specific *Pten*-deficiency in mice was shown previously to result in hepatic steatosis due to hyperactivated PKBβ in the liver. However, the role of peripheral insulin sensitive tissues on PTEN/PKBβ-dependent development of NAFLD has not been addressed.

The aim of this thesis is to characterize the effects of systemically perturbed PTEN/PKBβ signaling on hepatic lipid content using *Pten*-haplodeficient ($Pten^{+/-}/Pkb\beta^{+/+}$) mice and *Pten*-haplodeficient mice lacking *Pkbβ* ($Pten^{+/-}/Pkb\beta^{-/-}$). We found that $Pten^{+/-}/Pkb\beta^{+/+}$ mice have a more than 2-fold reduction in hepatic lipid content compared to control mice, similar to the low level observed in $Pten^{+/-}/Pkb\beta^{-/-}$ mice. $Pten^{+/-}/Pkb\beta^{+/+}$ mice showed enhanced insulin signaling in the liver indicating that extra-hepatic factors prevent hepatic lipid accumulation. Further results suggested that augmented PKBβ activity in the skeletal muscle of $Pten^{+/-}/Pkb\beta^{+/+}$ mice might reduce hepatic lipid content. Indeed, skeletal muscle-specific expression of constitutively active PKBβ reduced hepatic lipids in $Pten^{+/+}Pkb\beta^{+/+}$ mice and dominant negative PKBβ increased hepatic lipid content in both $Pten^{+/+}Pkb\beta^{+/+}$ and $Pten^{+/-}/Pkb\beta^{+/+}$ mice.

The results obtained during this study show that PKBβ activity in skeletal muscle regulates lipid accumulation in the livers of $Pten^{+/+}Pkb\beta^{+/+}$ and $Pten^{+/-}/Pkb\beta^{+/+}$ mice, and emphasizes the role of skeletal muscle in the pathophysiology of NAFLD.

1. Introduction

1.1. Global burden of obesity and diabetes

Obesity has now reached epidemic dimensions worldwide. The *World Health Organization* (WHO) estimates that there were 1.4 billion overweight (BMI = 25 – 29.9 kg/m^2) and 500 million obese (BMI ≥ 30 kg/m^2) people in the world in 2008 (Figure 1) (1). A study from 2005 projected that in 2030 there will be 2.2 billion overweight and 1.1 billion obese people (2). Even though recent data from the *Organisation for Economic Co-operation and Development* revealed that the rates of obesity is increasing less than previously projected and even remain stable in some countries, obesity will persist as a global burden in the future (3).

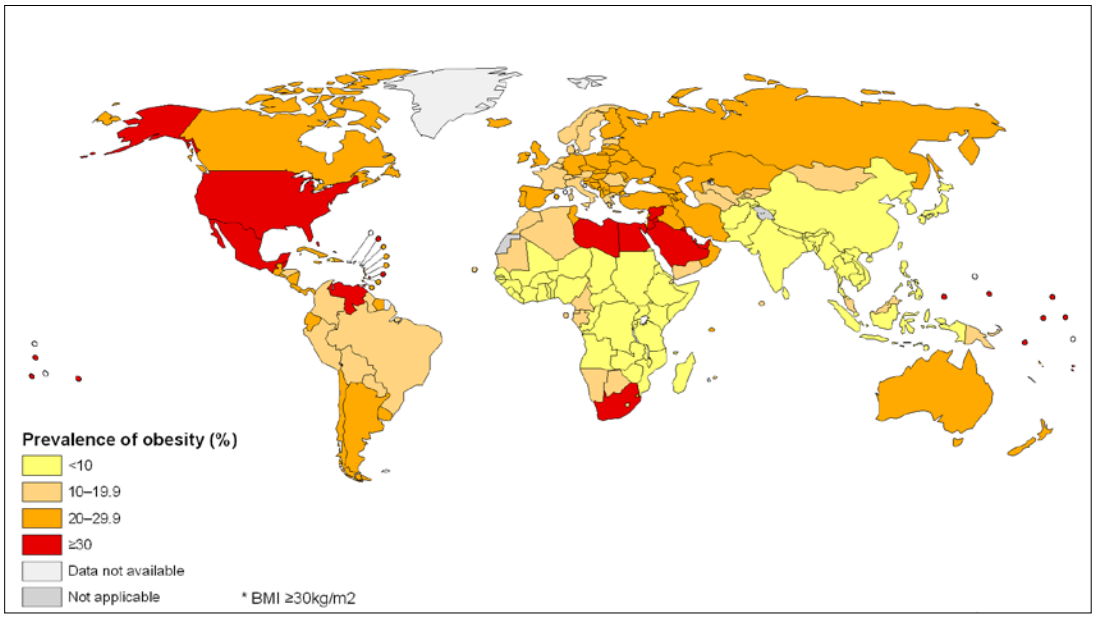

Figure 1. Global prevalence of obesity. The map is showing the global prevalence of obesity in people above an age of 20 years from both sexes in 2008. Image adapted from (4).

The increasing rates of overweight and obesity in children with a prevalence of 25% or higher in many countries are even more alarming (5, 6). Obese children may develop diseases such as cardiovascular disease and type 2 diabetes mellitus (T2D) and have increased risk of adult morbidity and premature mortality (5-7).

Obesity is the most common cause of insulin resistance and is the major risk factor for T2D (8, 9). Thus, the rising incidence of obesity is paralleled by an increasing number of insulin resistant and diabetic patients. It is estimated that there will be 439 million people with T2D in 2030 (10). Systemic inflammation and ectopic accumulation of lipids in cells are considered to drive insulin resistance in the context of obesity (8, 9, 11). The molecular mechanisms linking inflammation and intracellular lipids to insulin resistance are described in section *1.3.2. Mechanisms of obesity-induced / acquired insulin resistance*.

The clustering of obesity, insulin resistance and/or related comorbidities, such as hyperlipidemia, glucose intolerance and hypertension, increases the risk of developing cardiovascular disease and T2D and is known as the metabolic syndrome (12). It is now widely accepted that non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome (13).

1.2. Non-alcoholic fatty liver disease

1.2.1. NAFLD is a major health problem

NAFLD affects 30% of adults and 10% of children in the general population of developed countries (14-16). Importantly, NAFLD frequently occurs in the context of obesity, insulin resistance and T2D. Approximately 75% of obese and diabetic patients and 95% of morbid obese

(BMI $\geq$ 35 kg/m^2) patients develop NAFLD (17, 18). Given the high incidence of obesity and T2D, NAFLD is now the most common liver disease worldwide (19).

The term NAFLD defines the presence of hepatic steatosis in the absence of significant alcohol consumption and other liver diseases (20, 21). Traditionally, the term NAFLD has been used for a disease continuum from simple steatosis to steatohepatitis and fibrosis, but it was proposed to distinguish between simple steatosis (NAFLD) and more severe liver injuries (e.g. non-alcoholic steatohepatitis, NASH) (17). Hepatic steatosis is characterized by accumulation of predominantly macrovesicular lipid droplets in the cytoplasm of more than 5% of hepatocytes (20, 21). In early stages lipid droplets are typically clustered in acinar zone 3 and, in later stages, may occupy the whole acinus (Figure 2 A, B) (19, 22).

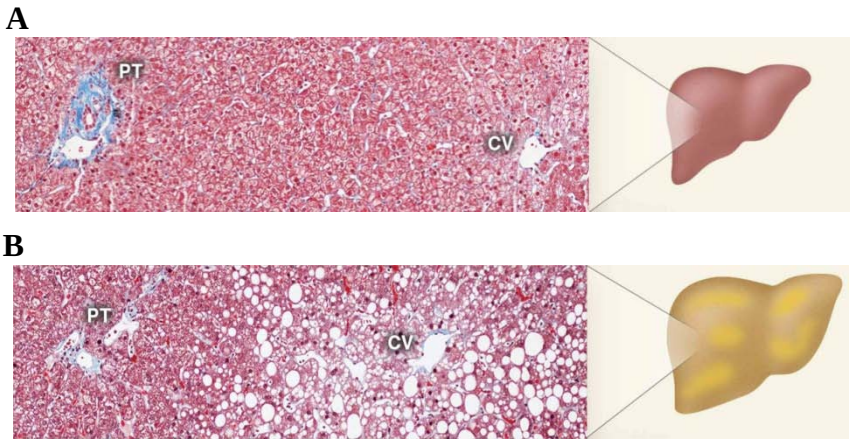

Figure 2. Appearance of hepatic steatosis. Histological sections show normal liver morphology (A) and accumulation of macro-vesicular lipid droplets in hepatocytes (B). PT, portal triad; CV, central vein. Image adapted from (23)

It is estimated that ~20% of obese patients with hepatic steatosis develop necro-inflammatory changes in the liver termed as NASH (13, 21). NASH may progress to fibrosis and cirrhosis and eventually to hepatic failure and hepatocellular carcinoma (13, 21, 23). As not all cases of NAFLD progress to NASH a "*2nd hit*" may be required to induce the progression of NAFLD. Today it is considered that a combination of multiple factors, such as genetic predisposition,

oxidative and endoplasmatic reticulum (ER) stress, hepatocyte death and higher susceptibility to liver damage by other means may trigger the progression of NAFLD to NASH (23-28). However, the individual contribution of these factors on disease progression remains controversial and is likely to differ from patient to patient. NASH may also not always be preceded by NAFLD (Figure 3) (17, 23). While in the past NAFLD was thought to be benign, it is now clear that it is a major health problem and, due to its forms of progression, it is about to become one of the primary indications for liver transplantation (23, 29).

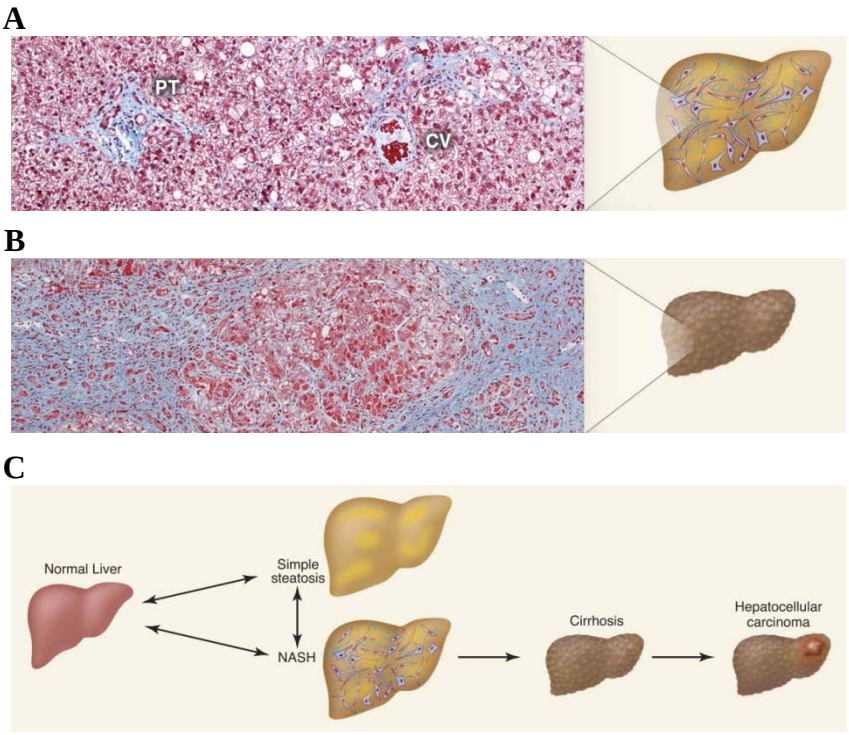

Figure 3. Progression of NAFLD to NASH. (A, B) Histological sections showing inflammatory changes in steatotic liver (A) and blue-stained fribrotic fibers in cirrhotic liver (B). (C) Schematic illustration of the progression from NAFLD to end stage liver diseases. PT, portal triad; CV, central vein. Images adapted from (23).

1.2.2. Development of NAFLD in the context of obesity and insulin resistance

The association of NAFLD with obesity and insulin resistance is well known, but the underlying mechanisms driving ectopic accumulation of lipids in hepatocytes are not fully understood.

In 2005, Donelly and colleagues characterized the relative contribution of different sources on hepatic lipid content in obese patients using a multi-stable-isotope approach (23, 29). This study revealed that in obese patients approximately 60% of lipids in the liver are derived from serum nonesterfied fatty acid (NEFA) pool, 25% originate from *de novo* lipogenesis in the liver and 15% are derived directly from dietary intake (23, 29). Given the high contribution of serum NEFA pool to hepatic lipid content and that the inhibitory effect of insulin on adipose tissue lipolysis is likely to be impaired in patients with NALFD, Donelly and colleagues concluded that adipose fatty acid flux is probably the major contributor to the development of NAFLD (29).

Several studies suggest that insulin resistance of skeletal muscle has a central role in the development of NAFLD. In these studies the metabolic fate of ingested carbohydrates was analyzed by protium (^{1}H) and carbon-13 (^{13}C) magnetic resonance spectroscopy in insulin resistant subjects (30, 31). The skeletal muscle glycogen synthesis in young, lean and insulin resistant subjects was reduced by approximately 60%, but hepatic *de novo* lipogenesis and triglyceride content were increased by more than 2-fold compared to insulin sensitive control subjects (30). Similar results were also seen in insulin resistant, elderly subjects (31). These studies indicate that muscle insulin resistance precedes hepatic insulin resistance and results in a redistribution of ingested carbohydrates from skeletal muscle glycogen synthesis towards hepatic *de novo* lipogenesis (30, 31). Importantly, hepatic *de novo* lipogenesis and lipid content were

found to be reduced by more than 30% after insulin sensitivity of skeletal muscle was improved by physical exercise in insulin resistant subjects (Figure 4) (32). Physical exercise improves insulin sensitivity and reduces hepatic lipid content also in obese and diabetic patients (33, 34). Thus, skeletal muscle insulin resistance could have a central role in NAFLD development and might be an effective therapeutic target (30-32).

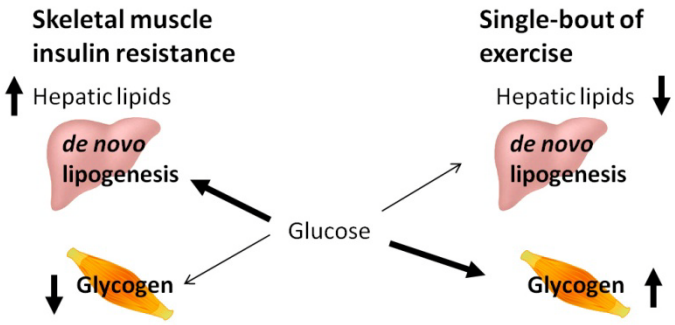

Figure 4. The effects of skeletal muscle insulin response on the fate of glucose. Skeletal muscle insulin resistance leads to redistribution of ingested glucose from glycogen synthesis in skeletal muscle towards hepatic *de novo* lipogenesis and increases hepatic lipid content. This can be inhibited by physical exercise. Image adapted from (23, 32).

Selective resistance of the hepatic insulin signaling could also contribute to ectopic accumulation of lipids in the liver (35, 36). According to this, insulin fails to inhibit gluconeogenesis but still induces *de novo* lipogenesis in hepatocytes (35). Insulin levels remain elevated due to steady output of glucose from the liver, which further boosts hepatic *de novo* lipogenesis and accumulation of lipids (Figure 5) (35). This concept is supported by the characterization of lipid metabolism in insulin resistant patients with primary defects in the insulin receptor (INSR) or with defects in the downstream protein kinase PKBβ/AKT2 (23, 36). While patients with defects in INSR have low hepatic lipid content and moderate rates of *de novo* lipogenesis, patients with defects in PKBβ display increased hepatic lipid content and the rate of *de novo* lipogenesis was increased by approximately 3-fold (36). However, the number of patients in this study is limited and the contribution of selective hepatic insulin resistance on NALFD development in obesity and acquired insulin resistance remains to be elucidated.

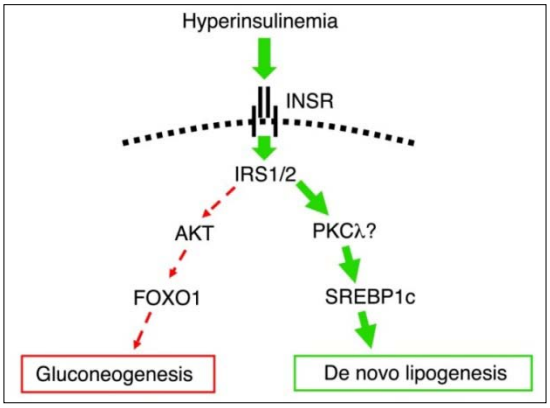

Figure 5. Model of selective insulin resistance in the liver. It is proposed that postreceptor insulin resistance affects only distinct processes such as gluconeogenesis but not *de novo* lipogensis. This could lead increased hepatic lipid content. Image adapted from (36).

While selective hepatic insulin resistance could contribute to NAFLD development, it is still a matter of debate whether accumulation of lipids in hepatocytes causes hepatic insulin resistance. Several studies in humans demonstrate a tight association between hepatic lipid content and insulin sensitivity. Patients with severe lipodysthrophy develop NAFLD and hepatic insulin resistance (37, 38). Treatment of these patients in with recombinant leptin resolves hepatic steatosis, which is accompanied by improved insulin sensitivity (37, 38). Diabetic patients that were placed on a hypocaloric diet displayed a marked reduction of hepatic lipid content and improved insulin sensitivity, despite the absence of changes in intramyocellular lipid content and peripheral glucose uptake (38, 39). Thus, it was concluded that accumulation of lipids in hepatocytes causes (hepatic) insulin resistance (38). However in a recent review, Cohen listed a number of studies in humans and mice with monogenetic defects that develop NAFLD without insulin resistance (23). For instance patients with mutations in *APOB* gene that impairs export of hepatic triglycerides in the form of very low density lipoprotein (VLDL) have elevated hepatic lipid content, but have insulin sensitivity similar to controls (23, 40). Mice with impaired lipid mobilization in the liver by knockdown of abhydrolase domain containing protein 5 (CGI-58) in the liver and adipose tissue have severe hepatic steatosis, but display improved glucose tolerance

and insulin sensitivity (23, 41). Moreover, Cohen points out that accumulation of specific lipid species such as diacylglycerol (DAG) and ceramides that are known to cause insulin resistance do not necessarily lead to insulin resistance in the liver of mice with hepatic steatosis (23). Thus, Cohen concluded that mere lipid accumulation in the liver is not the cause of hepatic insulin resistance (23). Most likely additional factors such as specific subcellular localization and composition of lipid species are required that increased lipid content in hepatocytes interferes with insulin action (23).

The proposed mechanisms driving lipid accumulation in the liver, the above-mentioned surplus in dietary energy intake, aberrant energy disposal due to peripheral and hepatic insulin resistance as well as elevated glucose and insulin level, are closely related and commonly coexist in obese and diabetic patients. Thus, it is likely that the development of NAFLD in the context of obesity and insulin resistance is a result of the combined action of these mechanisms (Figure 6).

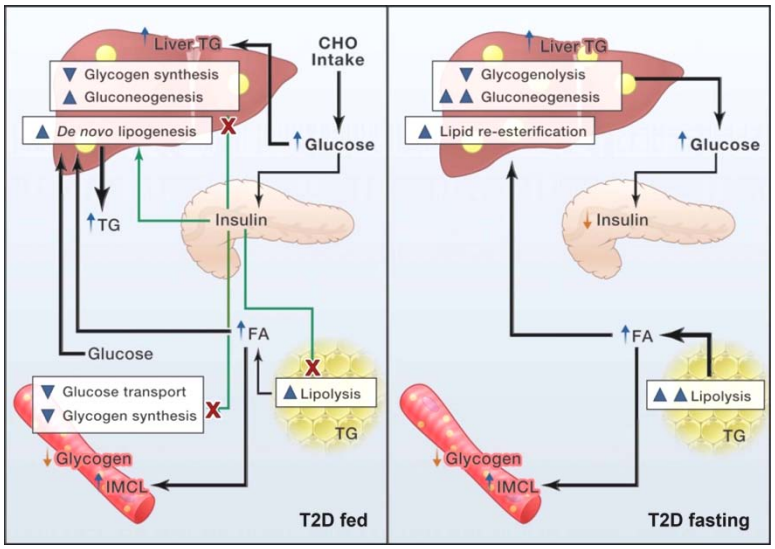

Figure 6. NAFLD development in the context of obesity and insulin resistance. A schematic overview is shown on how aberrant glucose disposal, selective insulin resistance and adipose tissue lipolysis contribute to NAFLD development during fed and fasted conditions in diabetic patients. CHO, carbohydrate; FA, fatty acids; IMCL, intramyocellular lipids; T2D, type 2 diabetes mellitus, TG, triglycerides. Image adapted from (38).

Unraveling the relative contribution of these mechanisms in NAFLD and characterizing the effects of (therapeutic) intervention on hepatic lipid content and whole-body metabolism is the basis for developing effective treatments of NAFLD.

1.3. Insulin signaling

1.3.1. The insulin/PI3K/PKB signaling pathway

Insulin is indispensable for the regulation of systemic metabolism by stimulating cellular glucose uptake and anabolic processes. T2D accounts for up to 95% of diagnosed diabetes cases and is characterized by impaired intracellular insulin signaling (postreceptor insulin resistance) (42).

Circulating insulin binds to the extracellular α-subunits of the heterotetrameric INSR, which induces conformational changes and facilitates autophosphorylation of tyrosine residues on the intracellular part of the membrane-spanning β-subunits of the INSR (43). Upon stimulation, INSR activates MAPK/ERK and PI3K/PKB signaling pathway (44). While activation of the MAPK/ERK pathway by insulin was found to be less critical, IRS1/2-depent activation of PI3K signaling is indispensable for regulation of metabolism by insulin (44).

The PI3K/PKB signaling pathway has been studied extensively and reviewed comprehensively elsewhere (44-47). An overview of the PI3K/PKB signaling pathway downstream of insulin is shown in Figure 7.

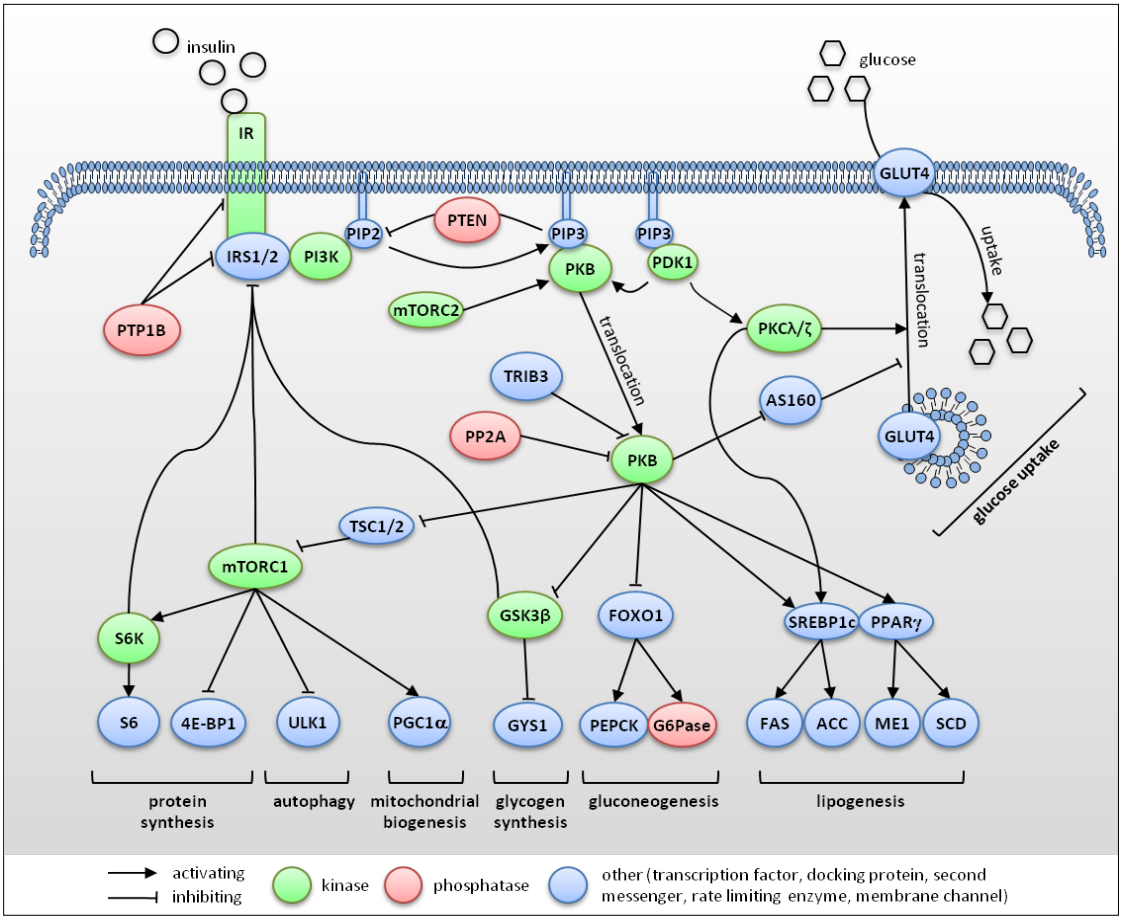

Figure 7. Simplified view of insulin-stimulated PI3K/PKB signaling and its substrates involved in cellular metabolism. The PI3K/PKB pathway is activated downstream of the INSR via binding of the regulatory subunit of PI3K (p85) to IRS1/2. This leads to recruitment and activation of the catalytic subunit of PI3K (p110). PI3K converts PIP2 to PIP3 at the plasma membrane. PKB binds via its PH-domain to PIP3, which facilitates activation of PKB by upstream kinases PDK1 and mTORC2. Upon activation, PKB phosphorylates GSK3β, FoxO1 and AS160, which regulate glycogen synthesis, gluconeogenesis and glucose uptake, respectively. PKB also activates mTORC1 by inhibiting TSC1/2. Activated mTORC1 upregulates mitochondrial biogenesis, inhibits autophagy and induces protein synthesis by regulation of PGC1α, ULK1 as well as S6K and 4E-BP1, respectively. PDK1 also activates PKCλ/ζ, which regulates glucose uptake. PKB and PKCλ/ζ regulate lipogenic genes, such as SREBP1-c and PPARγ. The insulin/PI3K/PKB pathway is negatively regulated by PTP1b, PTEN and PP2A that dephosphorylate and thereby inhibit the INSR, IRS1/2, PIP3 and PKB, respectively. PKB activity can also be inhibited by binding partners, such as TRB3. Negative feed-back loops are implemented to downregulate insulin signaling. GSK3β, mTORC1 and S6K can phosphorylate IRS on serine residues, which lead to ubiquitination and proteolytic breakdown. 4E-BP1, eIF4E-binding protein 1; AS160, AKT substrate 160; GLUT4, solute carrier family 2; GYS1, glycogen synthase; ME1, malic enzyme 1; PDK1, 3-phosphoinositide dependent protein kinase-1; S6, ribosomal protein S6; SCD, stearoyl-CoA desaturase; TRIB3, tribbles homolog; ULK1, unc-51-like kinase. Image and figure legend were adapted from (43).

The activity of PI3K/PKB signaling downstream of insulin is modulated by diverse physiological stimuli and insulin-independent mechanisms to adapt cellular insulin response to local nutrient and energy level. For instance, mTORC1 is part of the insulin signaling pathway regulating anabolic processes, such as cellular growth, mitochondrial biogenesis, protein synthesis and *de novo* lipogenesis (43). Upon insulin stimulation, PKB phosphorylates and inhibits TSC2 leading to accumulation of Rheb-GTP, which activates mTORC1 (48). mTORC1 is also activated by amino acids (49). In the presence of amino acids Rag GTPases are loaded with GTP (49). mTORC1 binds to the Rag GTP complex probably at lysomes facilitating its activation by Rheb-GTP (48, 49). Conversely, mTORC1 activity is inhibited at low cellular energy levels (49). A high AMP to ATP ratio, e.g. due to oxygen or glucose deprivation, activates AMP-activated protein kinase (AMPK). AMPK inhibits mTORC1 in two ways; by antagonizing Rheb-dependent activation of mTORC1 through stimulation of TSC2 and by phosphorylating Raptor that enables binding of 14-3-3 (48-50). These regulatory mechanisms indicate that mTORC1 has a central role in integrating systemic and cellular metabolism (Figure 8).

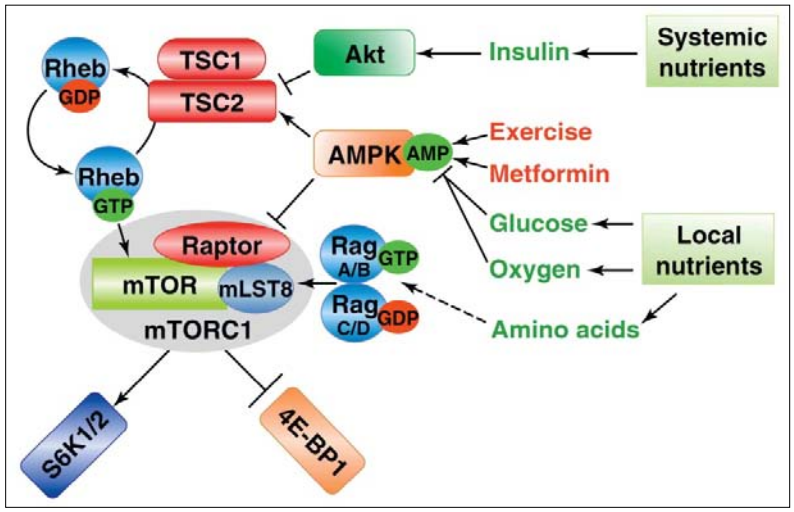

Figure 8. Insulin and insulin-independent regulation of mTORC1. Insulin activates mTORC1 in response to systemic nutrient levels. In addition, mTORC1 activity is dependent on local nutrient and energy status such as amino acids and oxygen level. Image adapted from (48).

mTORC2 is activated by growth factors, but the underlying mechanisms are poorly understood. Recently, it was shown that mTORC2 activity is dependent on its association with ribosomes, which is stimulated by insulin in a PI3K-dependent manner (51). It was proposed that this mechanism links mTORC2 activity to the growth capacity of the cell, which is determined by ribosomal content (51). Zanzilla et al showed that this mechanism has a functional role in tumorigenesis (51). It would be interesting to investigate the role of mTORC2-ribosome interaction in the regulation of systemic metabolism.

Insulin signaling is antagonized by stress kinases upon inflammation and cellular stress. In an evolutionary perspective this is beneficial by reserving nutrients for tissue repair or pathogen defense (52). However, this is also considered to be the underlying mechanism of obesity-induced / acquired insulin resistance leading to complications such as cardiovascular disease, NAFLD and T2D.

1.3.2. Mechanisms of obesity-induced / acquired insulin resistance

Inflammation and ectopic lipid accumulation in cells have a central role in the development of obesity-induced insulin resistance. They interfere with the action of insulin by different means, which can also be in an interconnected, synergistic manner.

Obesity is associated with a low-grade, chronic inflammatory state (53). Despite extensive research in this field, the etiology of low-grade inflammation is not yet fully understood. Several mechanisms such as lipotoxicity, higher permeability of the intestine for inflammatory

substances (e.g. liposaccharides) and an inflammatory nature of nutrients are proposed to trigger inflammation in the context of obesity (53, 54). Low-grade inflammation manifests predominantly in the adipose tissue, but also affects liver, skeletal muscle, pancreas and brain (53). It is characterized by infiltrating proinflammatory M1-like macrophages, mast cells, natural killer cells and T-cells leading to increased levels of cytokines such as tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and interleukin-1β (IL-1β) in affected tissues (8, 53). This inflammatory environment leads to activation of stress kinases such as JNK, IKKβ and PKCθ in adipocytes, myocytes and hepatocytes in a paracrine- as well as an endocrine-manner (8). The activation of JNK, IKKβ and PKCθ inhibits insulin signaling by phosphorylation and destabilization of IRS1 (8, 38, 53, 54). JNK and IKKβ also activate an inflammatory response in target cells (Figure 9) (8, 38, 54).

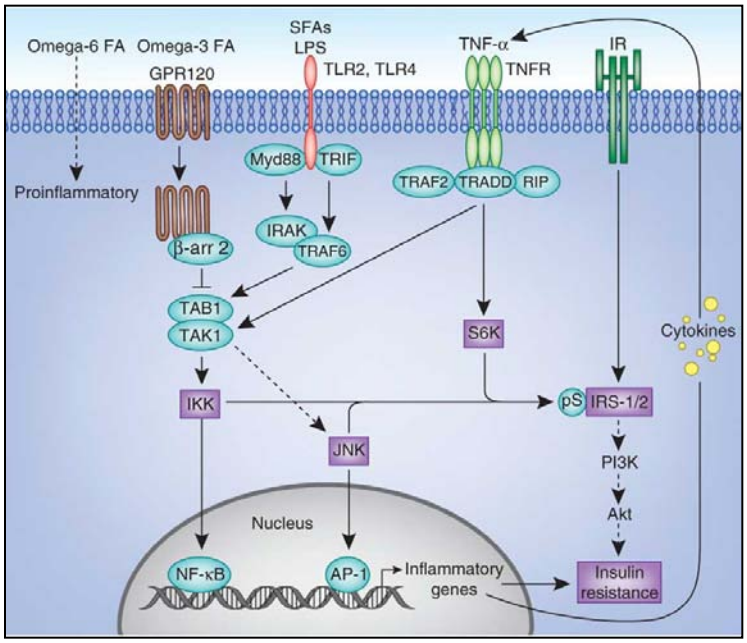

Figure 9. Insulin resistance due to elevated cytokine and free fatty acids concentrations. The activation of IKK and JNK downstream of cytokines and free fatty acids leads to insulin resistance by inhibition of IRS1/2 and the expression of inflammatory genes. Image adapted from (55).

Ectopic lipid accumulation in cells also interferes with insulin signaling by activating JNK and PKCθ in a direct and an indirect manner. Lipids, such as eicosanoids, phosphoinositides, ceramides and DAG are central components of intracellular signaling mechanisms and are involved in the regulation of cellular processes including metabolism (54). The intracellular levels of ceramids and DAG are known to be elevated in obese patients, in particular in myocytes and hepatocytes (38). DAG and ceramide directly interfere with insulin signaling by inhibiting IRS1/2 through PKCθ activation and by blocking of PKB via PP2A and PKCζ activation, respectively (Figure 10) (8, 54).

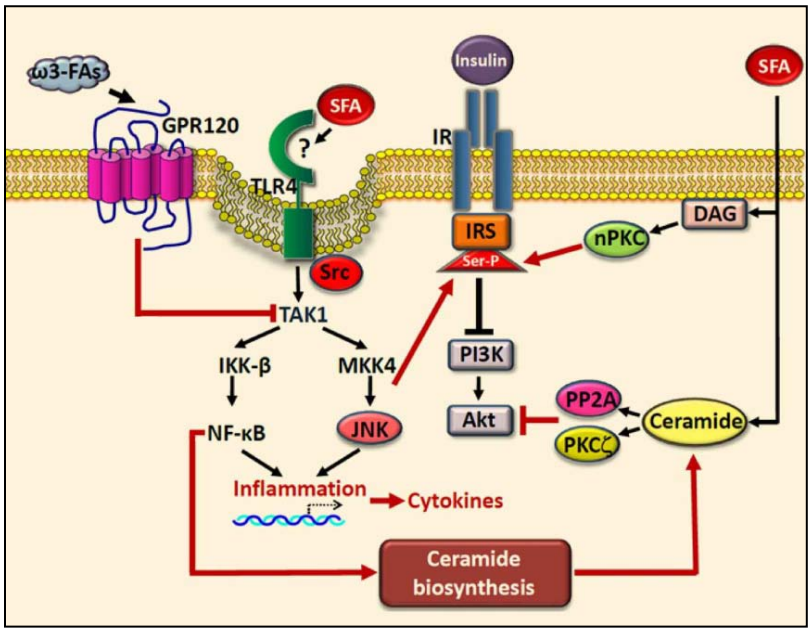

Figure 10. Inhibition of insulin action by DAG and ceramides. Intracellular DAG and ceramide concentrations are elevated by excessive energy intake and block insulin signaling by activation of nPKCs (e.g. PKCθ), PKCζ and PP2A. Image adapted from (8).

Intracellular accumulation of lipids can also lead to cellular stress by impairing the function of lipid-metabolizing organelles, such as ER and mitochondria (38, 54). Impaired ER function leads to oxidative stress by radical oxygen species (ROS) formation and activation of the unfolded protein response (UPR) (38, 54). Both, oxidative stress and UPR activate JNK, which blocks insulin signaling (38, 54). Oxidative and ER stress can additionally trigger inflammatory

response via JNK and IKKβ activation (53, 54). Circulating free fatty acids also provoke a inflammatory response by binding to Toll-like receptors (TLR), such as TLR2 and TLR4 (Figure 9 and 10) (54). For instance, bone marrow derived dendritic cells and pancreatic islets from TLR2-deficient mice had a diminished NEFA-induced inflammatory response (56). While body weights are similar to controls, TLR2-deficient mice displayed reduced tissue inflammation and an increase in energy expenditure accompanied by improved glucose tolerance and insulin sensitivity as well as reduced hepatic lipid content (56).

IL-1β has a major role in obesity-induced inflammation and insulin resistance and promotes the progression of insulin resistance to T2D by its toxic effects on β-cells (57, 58). Metabolic stress such as high glucose level and oxidative stress is sensed by the inflammasome NLR family, pyrin domain containing 3 (NLRP3) which induces secretion of IL-1β (57, 58). Notably, IL-1 receptor antagonists improve insulin sensitivity as well as function and survival of β-cells and have now been successfully used in the treatment of T2D (57-59).

1.4. Modified insulin signaling and its pathophysiology in mice

1.4.1. Mice with targeted deletion of genes implicated in insulin signaling

There is a high demand for new therapies to counter the increasing incidence of obesity, T2D and related comorbidities such as NAFLD. Mouse models have been used extensively to define the physiological role of effectors and regulators of the insulin signaling pathway in metabolism and related disorders. Examples of mouse models with targeted deletion of genes implicated in insulin signaling are shown in Table 1.

Gene	Deleted in	Insulin sensitivity	Glucose tolerance	Further characteristics	Refs
Ptp1b	whole body	+	+	protected against diet-induced diabetes	(60)
	skeletal muscle	+	+	protected against diet-induced insulin resistance	(61)
	hepatocytes	+	+	reduced hepatic lipid content after 5 weeks of a high fat diet; protected against diet-induced insulin resistance	(62)
Insr	whole body	nr	nr	neonatal mice display hyperglycemia and hyinsulenimia, develop ketoacidosis and die within 7 days after birth	(63)
	skeletal muscle	uc	uc	reduced skeletal muscle glucose uptake; elevated serum triglycerides and FFAs; enhanced adiposity	(64, 65)
	hepatocytes	-	-	hyperglycemic; hyperinsulemic; progressive hepatic dysfunction; atherosclerosis when fed with atherogenic diet	(66, 67)
	adipocytes	+	+	reduced fat mass; prolonged lifespan	(68, 69)
Irs1	whole body	-	IPGTT: - OGTT: uc	reduced body size	(70, 71)
Irs2	whole body	-	-	β-cell dysfunction	(72)
Pik3r1	whole body (only p85α)	+	+	loss of p85α only is compensated by p50α, which generates increased level of PIP3	(73)
	whole body (p50α, p55α, p85α)	nr	+	perinatal lethality; necrosis in liver and brown adipose tissue	(74)
Gsk3α	whole body	+	+	increased hepatic glycogen content; reduced adipose tissue mass	(75)
Gsk3β	whole body (-/-)	nr	nr	embryonic lethal	(76)
	whole body (+/-)	nr	nr	ameliorates genetically-induced diabetes	(76)
	panc β-cells	nr	+	increased pancreatic β-cell mass; protected against diet-induced diabetes	(77)
	hepatocytes	uc	uc	no distinct metabolic phenotype	(78)
	skeletal muscle	+	+	increased muscle glycogen content	(78)

Table 1. Mouse models with targeted deletion of genes implicated in insulin signaling. IPGTT, intraperitoneal glucose tolerance test; nr, not reported; OGTT, oral glucose tolerance test; panc, pancreatic; uc, unchanged; +, improved; -, reduced; (-/-), homozygous; (+/-), heterozygous. Table adapted from (43, 79).

Gene	Deleted in	Insulin sensitivity	Glucose tolerance	Further characteristics	Refs
Tsc1	panc β-cells	-	+	increased pancreatic β-cell mass; improved glycemic control in young mice; obesity in old mice	(80)
	hepatocytes	-	-	protected against diet-induced hepatic steatosis	(81, 82)
Tsc2	panc β-cells	nr	+	increased pancreatic β-cell mass	(83)
mTOR	skeletal muscle	uc	uc	increased muscle glycogen content; progressive muscle dystrophy; premature death	(84)
Rictor	hepatocytes	-	-	hypolipidemia; reduced hepatic lipid and glycogen content	(85)
	skeletal muscle	nr	nr	no phenotypical changes reported	(86)
Raptor	skeletal muscle	nr	-	increased muscle glycogen content; progressive muscle dystrophy; premature death	(86)
	adipocytes	nr	+	protected against diet-induced obesity and hypercholesterolemia	(87)
S6k	whole body	+	-	reduced pancreatic β-cell mass; hypoinsulinemia; protected against age- and diet-induced obesity and insulin resistance	(88, 89)

Table 1 (cont.). Mouse models with targeted deletion of genes implicated in insulin signaling. IPGTT, intraperitoneal glucose tolerance test; nr, not reported; OGTT, oral glucose tolerance test; panc, pancreatic; uc, unchanged; +, improved; -, reduced; (-/-), homozygous; (+/-), heterozygous. Table adapted from (43, 79).

These mouse models do not only reveal the role of respective genes in metabolic control, but also show that distinct modification of insulin signaling can be advantageous in normal as well as pathological conditions. For instance, PTP1b-deficient mice have improved glucose tolerance and insulin sensitivity and are protected against diet-induced insulin resistance most likely due to enhanced tyrosine phosphorylation of INSR and IRS1 (60). However, effectors of the insulin signaling pathway also act downstream of diverse growth factors regulating cell survival, proliferation and self-renewal. Thus, deletion of negative regulators of insulin signaling includes the risk of adverse effects such as tumorigenesis (43, 90).

The role of insulin response on metabolic control is highly dependent on the targeted tissue(s). Neonatal mice with whole-body INSR-deficiency have increased blood glucose and insulin concentrations, develop diabetic ketoacidosis and die within 7 days after birth (63). Liver-specific deletion of *Insr* in mice results in impaired glucose tolerance and insulin sensitivity and, when fed a high fat diet, these mice develop a severe atherosclerosis (66, 67). In contrast, mice with adipocyte-specific deletion of the *Insr* have reduced fat mass, are protected against age-related obesity and insulin resistance and were found to have a prolonged lifespan (68, 69). Thus, dependent on the targeted tissue, inhibition of insulin signaling can have a favorable or deteriorative outcome.

The effects of mTORC1-deficiency by deleting *Raptor* on systemic metabolism were also found to be dependent on the targeted tissues. While skeletal muscle-specific deletion of *Raptor* leads to progressive muscle dystrophy and glucose intolerance, mice with adipocyte-specific *Raptor*-deficiency were protected against diet-induced obesity and insulin resistance due to increased energy expenditure in adipocytes (43, 86, 91).

These findings indicate that activation as well as inhibition of insulin signaling in distinct tissues could be more advantageous than a systemic modification in the treatment of insulin resistance and related complications.

1.4.2. The role of PTEN and PKB in whole-body metabolism and NAFLD development

PTEN is frequently mutated in many types of cancer such as glioblastoma multiforme, prostate and breast cancer (92). PTEN acts as a tumor suppressor by antagonizing PI3K/PKB signaling via PIP3 dephosphorylation downstream of diverse growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (93). Recently, it was shown that PTEN has an additional, phosphatase-independent tumor suppressive function by inhibiting nuclear anaphase-promoting complex/cyclosome – CDC20 homologue 1 (APC-CDH1) complex (92, 94). As PTEN negatively regulates PI3K/PKB signaling, it also antagonizes insulin signaling. Interestingly, patients having *PTEN* mutations display improved insulin sensitivity as evidenced by 60% lower fasting insulin concentration and reduced insulin concentration by 67% in the area under the curve during an oral glucose tolerance test (95).

Homozygous deletion of *Pten* in mice causes embryonic lethality (96, 97). *Pten*-haplodeficient ($Pten^{+/-}$) mice are viable, but frequently develop tumors in liver, adrenal glands and the thyroid (96-98). Increased tumorigenesis was also observed in mice with tissue-specific deletion of *Pten* in pancreas, prostate and mammary glands (99-101). Notably, $Pten^{+/-}$ mice display an improved glucose tolerance and insulin sensitivity with enhanced glucose uptake in the skeletal muscle (102). Improved glycemic control was also observed in mice lacking PTEN specifically in pancreas, adipose tissue and skeletal muscle (Table 2) (103-105).

Gene	Deleted in	Insulin sensitivity	Glucose tolerance	Further characteristics	Refs
Pten	whole body (-/-)	nr	nr	embryonic lethal	(102)
	whole body (+/-)	+	+	protected against genetically-induced diabetes; spontaneous tumor development	(98, 102, 106)
	pancreas	nr	nr	hypoglycemia; hypoinsulinemia; protected against streptozotocin- and diet- induced diabetes	(103)
	skeletal muscle	uc	+	protected against diet-induced insulin resistance and diabetes	(105)
	adipocytes	+	+	resistant to streptozotocin-induced diabetes	(107)
	hepatocytes	nr	+	age-dependent hepatic steatosis and its progressive forms	(108, 109)

Table 2. Overview of mouse models for PTEN. nr, not reported; uc, unchanged; +, improved; -, reduced; (-/-), homozygous; (+/-), heterozygous. Table adapted from (43).

Importantly, mice with a hepatocyte-specific deletion of *Pten* show spontaneous accumulation of hepatic lipids starting at 10 weeks of age and develop severe hepatic steatosis and hepatocellular carcinoma in an age-dependent manner (108, 109). Overall, the reported phenotype is similar to the pathology of human NAFLD and its forms of progression. It was proposed that hepatic lipid accumulation is due to hepatocyte-intrinsic processes such as increased *de novo* lipogenesis driven by hyperactivated PKBβ (108, 109). In line with these findings, mice that overexpress *Pten* were shown to be protected against diet-induced hepatic steatosis (110). The authors proposed that enhanced energy expenditure in the brown adipose tissue improves metabolic control (110). The protection against hepatic steatosis, however, could also be due to diminished PKB activation in the liver, which was not addressed in this study.

The PKB serine/threonine protein kinase family consists of three evolutionary conserved isoforms (111). PKBα (AKT1), PKBβ (AKT2) and PKBγ (AKT3) are encoded by individual

genes and located on different chromosomes (111). The amino acid sequence of PKB isoforms is identical by approximately 80% and they form the same protein structure, including a N-terminal pleckstrin homology (PH), a catalytic and a C-terminal regulatory domain (43, 112). PKBβ is considered to be the major isoform downstream of the insulin receptor. Remarkably, a mutation in the kinase domain of PKBβ (arginine to histidine at position 274, R274H) that greatly reduces kinase activity of PKBβ was identified in a family with autosomal dominant inherited severe insulin resistance (113). $PKB\beta^{R274H}$ was shown to act in a dominant-negative manner in that its overexpression blocks the inhibition of forkhead box protein A2 (FOXA2) in HepG2 cells and impairs adipocyte differentiation *in vitro* (43, 113). Conversely, patients with constitutive activation of PKBβ due to a mutation in the PH-domain (E17K) have severe fasting hypoglycemia (114).

Mice lacking PKBγ do not show metabolic alterations (115, 116). PKBα-deficient mice apparently have improved glycemic control and are protected against diet-induced obesity due to enhanced energy expenditure (Table 3) (115, 117). In line with the role of PKBβ in humans, *Pkbβ*-deficient mice develop severe insulin resistance and a diabetes mellitus-like syndrome due to hepatic and skeletal muscle insulin resistance (118, 119).

Pkbβ-deficiency was found to protect against genetically- and diet-induced NAFLD. Whole-body as well as hepatocyte-specific deletion of *Pkbβ* inhibits the development of hepatic steatosis in mice with hepatocyte-specific *Pten*-deficiency, *leptin*-deficient mice and mice fed a high fat diet (120-122).

Gene	Deleted in	Insulin sensitivity	Glucose tolerance	Further characteristics	Refs
Pkbα	whole body	+	+	reduced body size; increased neonatal mortality; protected against diet-induced obesity and insulin resistance	(115, 123)
	skeletal muscle	nr	nr	not protected against diet-induced obesity	(117)
	brain	nr	nr	not protected against diet-induced obesity	(117)
Pkbβ	whole body	-	-	diabetes-like phenotype with compensatory increase in pancreatic β-cell mass; protected against genetically- and diet-induced hepatic steatosis	(115, 118, 120, 122, 124)
	hepatocytes	nr	nr	protected against genetically- and diet-induced hepatic steatosis	(122)
Pkbα/ Pkbβ	hepatocytes	nr	-	constitutive active FoxO1 impairs adaption to fasted and fed conditions	(125)
Pkbγ	whole body	uc	uc	impaired postnatal brain development; no obvious metabolic phenotype	(115, 116)

Table 3. Overview of mouse models for PKB isoforms. nr, not reported; uc, unchanged; +, improved; -, reduced. Table adapted from (43).

Loss of PKBβ leads to downregulation of lipogenic genes such as SREBP-1c, FAS and ACC and reduced *de novo* lipogenesis in the liver (120, 122). But the regulation of lipogenic genes by PKBβ is also context-dependent. While in mice with hepatocyte-specific *Pten*-deficiency and *leptin*-deficiency the expression of lipogenic genes was found to be dependent on PKBβ, the expression was not altered in *Pkbβ*-deficient mice fed with normal chow or a high-fat diet enriched in simple carbohydrates (Surwit diet) (109, 120, 122).

The accumulation of lipids in the liver does not only depend on hepatic PTEN and PKBβ but, as shown in the following sections, also on PTEN and PKBβ activity in skeletal muscle via systemic interactions.

2. Scope of the thesis

Previously, it was shown that accumulation of lipids in the liver of mice with hepatocyte-specific deletion of *Pten* depends on hepatic PKBβ. But the role of PTEN and PKBβ in peripheral insulin sensitive tissues on accumulation of hepatic lipids was not addressed. The aim of this thesis is to characterize the effects of systemically perturbed PTEN/PKBβ signaling on accumulation of lipids in the liver.

To this end we used mice with whole-body *Pten*-haplodeficiency ($Pten^{+/-}/Pkb\beta^{+/+}$) that have reduced PTEN level in all tissues such as liver, pancreas, adipose tissue and skeletal muscle. *Pten*-haplodeficient mice lacking PKBβ ($Pten^{+/-}/Pkb\beta^{-/-}$) were used to dissect the role of PKBβ in this mouse model. The liver, pancreas, adipose tissue and skeletal muscle were characterized by histology and/or the analysis of insulin signaling by Western blotting and quantitative real-time PCR (qRT-PCR). To assess the effects of PKBβ activity in skeletal muscle on hepatic lipid content, PKBβ mutants were expressed in skeletal muscle of $Pten^{+/+}/Pkb\beta^{+/+}$ and $Pten^{+/-}/Pkb\beta^{+/+}$ mice using adeno-associated virus 8 as a vector.

The present study shows that hepatic lipid content is reduced by 2-fold in $Pten^{+/-}/Pkb\beta^{+/+}$ compared to control mice despite increased activation of PKBβ and upregulation of lipogenic genes in the liver. We found that an enhanced skeletal muscle insulin response mediated by PKBβ reduces the accumulation of hepatic lipids in both $Pten^{+/+}/Pkb\beta^{+/+}$ and $Pten^{+/-}/Pkb\beta^{+/+}$ mice. Our results support the notion that skeletal muscle insulin resistance is a central factor in the development of NAFLD. Thus, improving the insulin response in skeletal muscle by physical exercise and/or insulin sensitizer may be an effective option for treatment of NALFD.

3. Results

3.1. General notes

The results obtained during my thesis are shown in the following manuscript entitled "AKT2/PKBβ activation in skeletal muscle regulates hepatic lipid content in *Pten*-haplodeficient mice".

Parts of the text in the manuscript were taken from the summary, introduction, scope of the thesis and general discussion of this thesis.

The numbering of references and figures of the manuscript is separate to that from the introduction and general discussion meaning that the first reference and the first figure of the manuscript is numbered as "1".

3.2. List of contributions to the manuscript

Oliver Tschopp and **Markus Niessen** were involved in all steps of this project including study design, acquisition of data, analysis and interpretation of data and manuscript writing.

Andreas Geier, Giagten A. Spinas and **Brian A. Hemmings** supervised the project and critically revised the manuscript.

Debby Hynx assisted *in vivo* work by supporting colony maintenance, measurement of blood glucose level and tissue sampling.

Heidi Seiler and **Sandrine Bichet** assisted the staining of histological sections.

Laurent Gelman, Aaron Ponti, Steve Bourke, Arno Doelemeyer and **Patrick Schwarb** supported image acquisition, quantitative analysis of histological specimen and gave technical advice.

Josephine Juettner taught me how to produce adeno-associated virus 8 and gave technical advice**.**

Patrick King edited the manuscript.

I was substantially involved in all steps of this project including study design, acquisition of data, analysis and interpretation of data, figure preparation and manuscript writing.

3.3. AKT2/PKBβ activation in skeletal muscle regulates hepatic lipid content in *Pten*-haplodeficient mice

AKT2/PKBβ activation in skeletal muscle regulates hepatic lipid content in *Pten*-haplodeficient mice

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Short title: AKT2 in skeletal muscle regulates hepatic lipids

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Abbreviations: AAV, adeno-associated virus; AUC, area under the curve; DAPI, 4′,6-diamidin-2-phenylindo; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation; T2D, type 2 diabetes mellitus; TG, triglycerides

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Disclosures: The authors disclose no conflicts.

Author Contributions: O.T., S.M.S. and M.N. were involved in all steps of the project, including study design, acquisition of data, analysis and interpretation of data and manuscript writing. D.H. assisted *in vivo* work. A.G., G.A.S. and B.A.H. supervised the project and critically revised the manuscript.

Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a major health problem and occurs frequently in the context of metabolic syndrome and type 2 diabetes mellitus. Hepatocyte-specific *Pten*-deficiency in mice was shown previously to result in hepatic steatosis due to hyperactivated AKT2. However, the role of peripheral insulin-sensitive tissues on PTEN- and AKT2-dependent development of NAFLD has not been addressed.

Methods: Effects of systemically disturbed PTEN/AKT2 signaling on hepatic lipid content were studied in *Pten*-haplodeficient ($Pten^{+/-}/Akt2^{+/+}$) mice and *Pten*-haplodeficient mice lacking *Akt2* ($Pten^{+/-}/Akt2^{-/-}$). The liver and skeletal muscle were characterized by histology and/or the analysis of insulin signaling. To assess the effects of AKT2 activity in skeletal muscle on hepatic lipid content, AKT2 mutants were expressed in skeletal muscle of $Pten^{+/+}/Akt2^{+/+}$ and $Pten^{+/-}/Akt2^{+/+}$ mice using adeno-associated virus 8 as vector.

Results: $Pten^{+/-}/Akt2^{+/+}$ mice were found to have a more than 2-fold reduction in hepatic lipid content, at a level similar to that observed in $Pten^{+/-}/Akt2^{-/-}$ mice. Insulin signaling in the livers of $Pten^{+/-}/Akt2^{+/+}$ mice was enhanced, indicating that extra-hepatic factors prevent lipid accumulation. The skeletal muscle of $Pten^{+/-}/Akt2^{+/+}$ mice also showed enhanced insulin signaling. Skeletal muscle-specific expression of constitutively active AKT2 reduced hepatic lipid content in $Pten^{+/+}/Akt2^{+/+}$ mice, and dominant negative AKT2 led to an increase in accumulation of hepatic lipids in both $Pten^{+/+}/Akt2^{+/+}$ and $Pten^{+/-}/Akt2^{+/+}$ mice.

Conclusion: The results of this study demonstrate that AKT2 activity in skeletal muscle critically affects lipid accumulation in the livers of $Pten^{+/+}/Akt2^{+/+}$ and $Pten^{+/-}/Akt2^{+/+}$ mice, and emphasize the role of skeletal muscle in the pathophysiology of NAFLD.

Keywords: NAFLD; liver; metabolism

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major complication in patients with metabolic syndrome and type 2 diabetes mellitus (T2D) and affects approximately one-third of adults and about 10% of children in developed countries [1-3]. NAFLD is characterized by the accumulation of predominantly macrovesicular lipid droplets in the cytoplasm of hepatocytes [4]. A substantial number of patients with NAFLD develop necro-inflammatory changes in the liver (non-alcoholic steatohepatitis, NASH), which may lead to cirrhosis and eventually to hepatocellular carcinoma (HCC) and hepatic failure [4,5]. Diverse factors, such as oxidative and endoplasmatic reticulum stress and hepatocyte death have been proposed to trigger progression of NAFLD to NASH, but the individual impacts of these factors remain controversial [6-10]. Although the association of NAFLD with insulin resistance and T2D is well known, the molecular mechanisms have not been fully elucidated. It is considered that insulin resistance of skeletal muscle and/or selectively impaired hepatic insulin signaling drive ectopic lipid accumulation in the liver [11-13].

Insulin is indispensable for the regulation of systemic metabolism. It stimulates cellular glucose uptake and induces anabolic processes, largely mediated by AKT [14]. PTEN negatively regulates insulin signaling by antagonizing activation of AKT [14]. PTEN and AKT also act downstream of several other stimuli and are pivotal regulators of elementary cellular processes such as proliferation, differentiation, survival and cell growth [15]. Consequently, deregulation of PTEN and/or AKT often results in disease, such as cancer and neurodegeneration [16,17]. Stimuli- and context-specificity of AKT are, at least partially, mediated by the recruitment of different isoforms of AKT (AKT1/PKBα, AKT2/PKBβ, AKT3/PKBγ) [18,19]. AKT2 is

considered to be the major isoform downstream of the insulin receptor and mice lacking *Akt2* develop severe insulin resistance and a T2D-like syndrome due to hepatic and skeletal muscle insulin resistance [20,21].

PTEN-deficiency results in the hyperactivation of AKT. Whilst homozygous deletion of *Pten* in mice causes embryonic lethality, *Pten*-haplodeficient ($Pten^{+/-}$) mice are viable [22]. Such mice show improved glucose tolerance and insulin sensitivity, but aged $Pten^{+/-}$ mice frequently develop tumors in liver, colon and thyroid glands [22,23]. Importantly, mice with a hepatocyte-specific deletion of *Pten* show spontaneous accumulation of hepatic lipids starting at 10 weeks of age and severe hepatic steatosis and HCC develop in an age-dependent manner [24,25]. Overall, the reported phenotype is similar to the pathology of human NAFLD and its forms of progression. It was shown that hepatic lipid accumulation in these mice is driven by hyperactivated AKT2 in a hepatocyte-autonomous manner [24-26].

However, the metabolic state of the liver also depends on systemic metabolism, which is regulated by multiple insulin-sensitive tissues. The physiological role of peripheral insulin-sensitive tissues in PTEN/AKT2-dependent development of NAFLD has not been addressed. In the present study, mice with whole-body *Pten*-haplodeficiency ($Pten^{+/-}/Akt2^{+/+}$) were used to analyze the impact of metabolically relevant tissues on hepatic lipid content. *Pten*-haplodeficient mice lacking *Akt2* ($Pten^{+/-}/Akt2^{-/-}$) were used to dissect the role of AKT2 in this mouse model.

In contrast to hepatic steatosis reported in mice with a hepatocyte-specific deletion of *Pten*, we show here that the hepatic lipid content of $Pten^{+/-}/Akt2^{+/+}$ mice is more than 2-fold lower than

that of *Pten*$^{+/+}$/*Akt2*$^{+/+}$ control mice and at a level similar to that observed in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice. In contrast to the reduced lipid content, *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice showed enhanced insulin signaling in the liver, in line with the notion that extra-hepatic factors prevent lipid accumulation in the livers of these mice. Analyses of peripheral insulin-sensitive tissues indicated that enhanced AKT2 activation in skeletal muscle reduces hepatic lipid accumulation in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. Significantly, skeletal muscle-specific expression of constitutively active AKT2 reduced hepatic lipid accumulation in *Pten*$^{+/+}$/*Akt2*$^{+/+}$ mice and dominant negative AKT2 led to increases in hepatic lipid content in both *Pten*$^{+/+}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice.

Material & Methods

Mice

All animal experiments were performed in accordance with Swiss Federal Animal Regulations and approved by the Veterinary Office of Zurich and Basel, Switzerland. Mice with whole-body targeted deletion of *Pten* and *Akt2* were described previously and were in a C57BL/6 background after at least 6 backcrosses [21,27]. $Pten^{+/+}/Akt2^{+/+}$, $Pten^{+/-}/Akt2^{+/+}$ and $Pten^{+/-}/Akt2^{-/-}$ mice were obtained by crossing $Pten^{+/-}/Akt2^{+/-}$ mice. The experimental mice were 20- to 22-week-old males. Mice were fasted by removing food for 8 h during the dark cycle. Fasted-refed mice were refed for 2 h after 8 h of fasting. Insulin stimulation was performed in fasted and terminally anesthetized mice by injection of human recombinant insulin at 1 U / kg of body weight (Novo Nordisk, Kuesnacht, Switzerland) via the inferior vena cava; samples were collected after 20 min. Mice were housed in groups with a 12-h dark-light cycle and free access to food and water, unless otherwise indicated.

Vector production and administration

Adeno-associated virus (AAV) 8 vectors were generated by triple plasmid transfection of HEK293T cells using jetPEI (Polyplus, Illkirch, France). AAV transplasmid (serotype 8) and helper plasmid were obtained from Penn Vector Core, Philadelphia, PA. myr-AKT2 and $AKT2^{K180A}$ were cloned as described previously and cloned into AAV expression vector containing a CMV promoter and a 2A-GFP reporter gene [28,29]. Viral particles were purified using a discontinuous iodixanol gradient as previously described [30]. Titers were determined by quantitative real-time polymerase chain reaction (qRT-PCR). 2×10^{11} genome copies of AAV8 viral particles were administered to 4-day-old mice by intraperitoneal injection. Skeletal muscle-

specific transgene expression results from selective retention of the vector DNA [31]. Mice used for subsequent metabolic analyses were 18- to 20-week-old.

Analysis of metabolic parameters in blood and tissues

Glucose levels were measured in tail vein blood using a glucose meter Freestyle (Disetronic, Burgdorf, Switzerland). Glucose tolerance tests were performed with fasted mice by intraperitoneal injection of 2 g D-(+)-glucose anhydrous / kg of body weight (Fluka, Buchs, Switzerland) and glucose levels measured at indicated time points. Triglyceride contents of skeletal muscle and liver and glycogen contents of skeletal muscle were determined as described previously [32].

Histology and quantitative analysis

Hematoxylin and eosin (H&E) and Oil Red O (Sigma-Aldrich, Saint Louis, MO) staining were performed according to standard protocols on paraffin and frozen sections, respectively. For fluorescent staining of lipids, frozen sections were fixed in 10% formaldehyde, incubated with 1 µg/ml BODIPY493/503 (Invitrogen, Carlsbad, CA) in 150 mM NaCl for 20 min at room temperature, and counterstained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Saint Louis, MO). BOPIPY493/503-stained areas relative to total tissue areas were quantified using Imaris software (Bitplane, Zurich, Switzerland). GFP staining was performed using Ventana DiscoveryXT (Roche Diagnostics, Mannheim, Germany) with a customized procedure for fluorescent staining. Slides were pre-treated with mild CC1, incubated with anti-GFP antibody (Invitrogen, Carlsbad, CA) for 1 h at 37°C, incubated with goat anti-

rabbit conjugated with Alexa fluor 647 (Invitrogen, Carlsbad, CA) for 32 min at 37°C and counterstained manually with DAPI.

Western blot analysis

Western blot analysis was performed using standard protocols (GE Healthcare, Buckinghamshire, UK). Images were captured on film or by BioSpectrum Imaging System (UVP, Cambridge, UK). Signal intensities were quantified by photodensitometry after background subtraction relative to β-Actin and normalized to fasted $Pten^{+/+}/Akt2^{+/+}$ mice. Antibodies against the following proteins were used: PTEN (Nicholas K. Tonks, Cold Spring Harbor Laboratory, USA), PTEN, pan-AKT, AKT1, AKT2, p-AKT S473, p-AKT T308, GSK3α/β, p-GSK3β, FoxO1, p-FoxO1 (Cell Signaling, Beverly, MA) and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Quantitative real time PCR

Total RNA was isolated from tissues using TRIZOL (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. cDNA synthesis was performed with M-MuLV reverse transcriptase (New England BioLabs, Ipswich, MA) according to the manufacturer's instructions. qRT-PCR reactions were performed using SYBR Green (Invitrogen, Carlsbad, CA) on ABI Prism 7000 or StepOnePlus Real-Time PCR System (Applied Biosystems, Rotkreuz, Switzerland). Primer sequences were obtained from PrimerBank [33]. The primers used and the corresponding PrimerBank ID were *Acc* (ID: 14211284a1), *Fas* (ID: 30911099a2), *G6Pase* (ID: 31982353a1), *Gck* (ID: 31982798a1), *Pepck* (ID: 7110683a1), *Pgc1a* (ID: 238018130b1), *Ppara* (ID: 31543500a1), *Pparg* (ID: 6755138a2) and *Srebp-1c* (ID: 14161491a1).

Statistical analysis

All data are presented as means ± standard deviation (SD). Data were subjected to Student's *t*-test for statistical significance (*P <0.05; **P <0.01). The numbers of independent biological samples per group used for each analysis are indicated accordingly.

Results

Improved glucose homeostasis in Pten$^{+/-}$/Akt2$^{+/+}$ mice depends on AKT2

Male *Pten*$^{+/+}$*/Akt2*$^{+/+}$, *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice 20- to 22-week-old were used to analyze the effects of systemically perturbed PTEN and AKT2 signaling on hepatic lipid content. Western blot analysis of PTEN, AKT2 and AKT1 protein levels in liver, skeletal muscle and adipose tissue was performed to validate our mouse model (Fig. 1A, B, Fig. S1).

Pten$^{+/-}$ mice were reported previously to be slightly hypoglycemic with improved glucose tolerance, whereas *Akt2*$^{-/-}$ mice were hyperglycemic and glucose intolerant [20,23,34,35]. Body weights and fasting blood glucose concentrations of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice were similar to controls (data not shown, Fig. 1C). However, when randomly fed or refed after fasting, *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice showed a reduction and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice an increase in blood glucose concentrations (Fig. 1 D, E). We performed glucose tolerance tests by intraperitoneal injection of glucose to further assess glycemic control. The glucose tolerance of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice had improved significantly compared to *Pten*$^{+/+}$*/Akt2*$^{+/+}$ control mice (area under the curve -22.2% ± 18.8%; $P < 0.05$) (Fig. 1F). Interestingly, glucose tolerance of *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice was similar to control mice, indicating compensation of acute glucose challenges (Fig. 1F).

These data verify the efficacy of gene targeting in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice and also show that the reduced blood glucose concentration and improved glucose tolerance of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice are dependent on AKT2 activity.

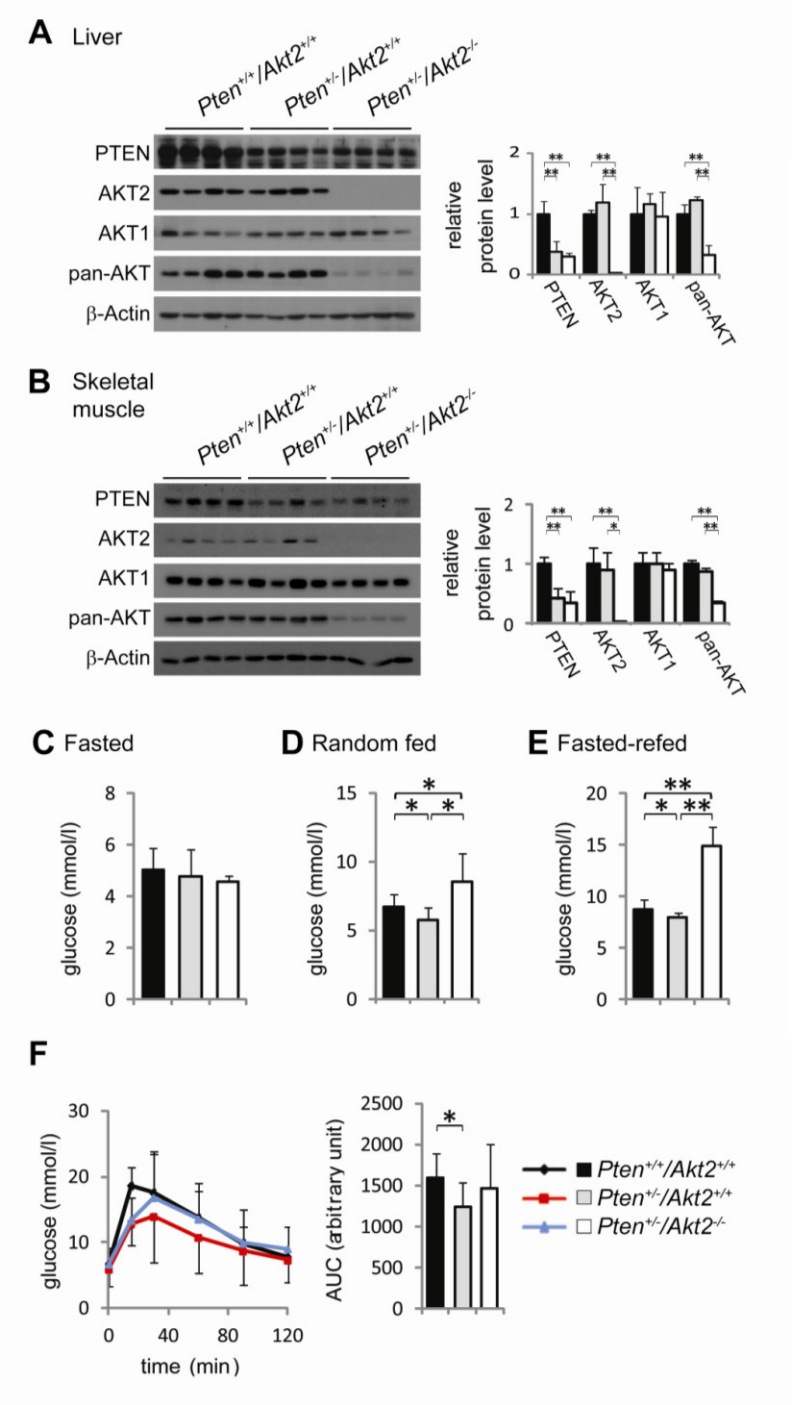

Figure 1. Improved glycemic control in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice is dependent on AKT2. (A, B) Protein levels of PTEN, AKT2, AKT1 and pan-AKT in liver (A) and skeletal muscle (B) analyzed by Western blot. Densitometric quantification is shown. *n* = 4/group. (C, D, E) Blood glucose concentrations from fasted (C), random fed (D) and fasted-refed (E) mice. *n* = 5-9/group. (F) Blood glucose concentrations in fasted mice after intraperitoneal administration of glucose were measured at the indicated time points; respective AUCs are shown. *n* = 7-8/group. AUC, area under the curve; data are expressed as means ± SD; * *P* <0.05; ** *P* <0.01.

Hepatic lipid content is reduced in both Pten$^{+/-}$/Akt2$^{+/+}$ and Pten$^{+/-}$/Akt2$^{-/-}$ mice

Mice with a hepatocyte-specific *Pten*-deficiency spontaneously develop hepatic steatosis [24,25]. Here we analyzed the livers of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice to examine the effects of systemically perturbed PTEN/AKT2 signaling on hepatic lipid content.

In contrast to the hepatomegaly reported in mice with hepatocyte-specific *Pten*-deficiency, *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice displayed only minor changes in liver weights (Fig. 2A) [24,25]. Histological analysis of liver sections was performed to examine the accumulation of hepatic lipids. Hepatic steatosis was not observed in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice (Fig. 2B).

In particular, lower amounts of lipids were found in liver sections of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice stained with Oil Red O and BODIPY493/503 compared to *Pten*$^{+/+}$/*Akt2*$^{+/+}$ control mice (Fig. 2C, D). The quantification of BODIPY493/503-stained area and measurements of hepatic triglycerides confirmed that hepatic lipid content in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice is reduced by more than 2.3-fold (Fig. 2E, F).

Thus, in contrast to mice with a hepatocyte-specific deletion of *Pten*, the hepatic lipid content in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice was reduced to levels similar to those observed in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice.

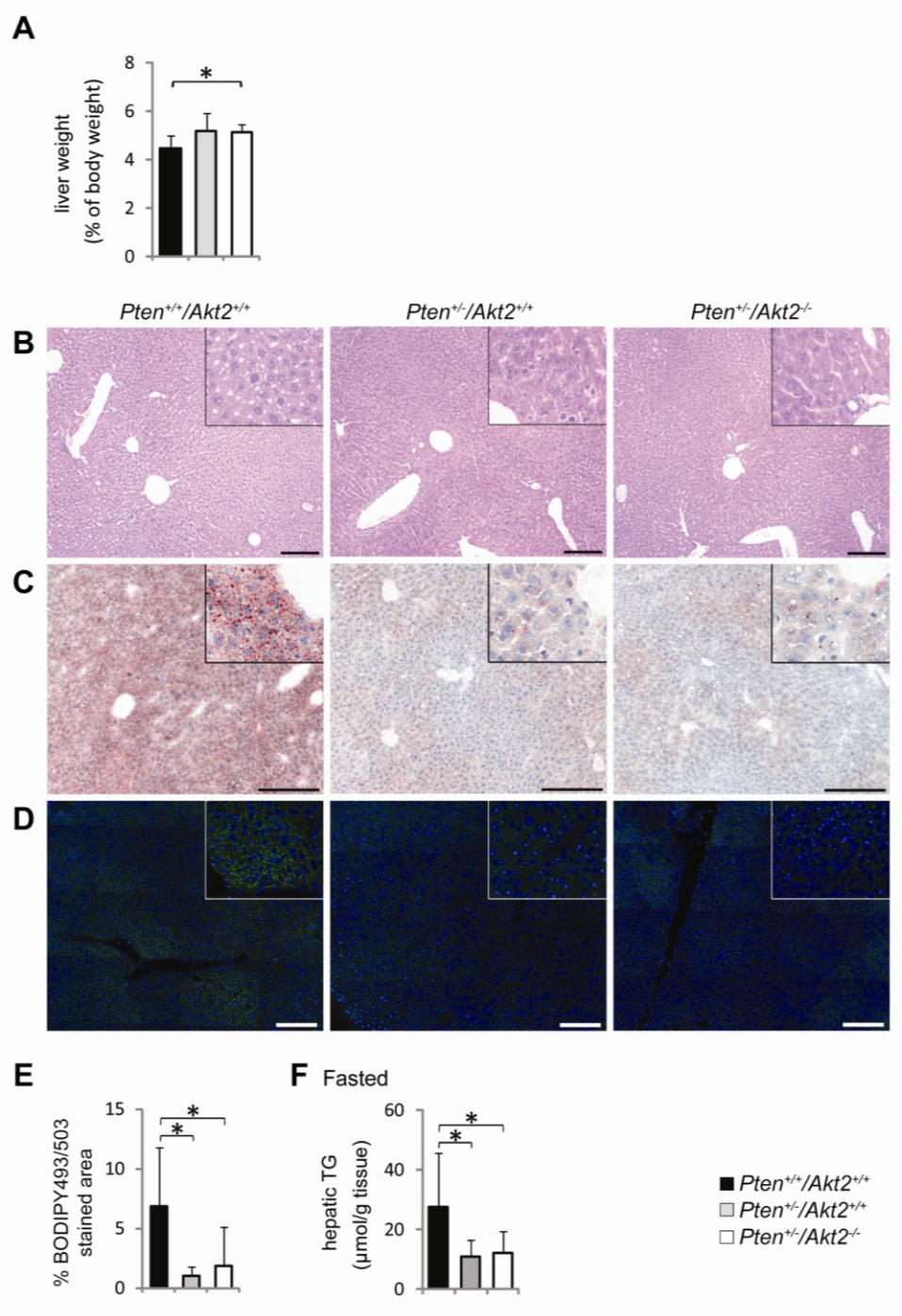

Figure 2. Reduced hepatic lipid content in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice. (A) Liver to body weight ratios. (B, C, D) Representative images of liver sections from fasted mice stained with H&E (B), stained for lipids with Oil Red O (C), and stained for lipids and DNA with BODPIY493/503 (green) and DAPI (blue), respectively (D). Image inlays show part of the depicted area at higher magnification. (E) Relative BODIPY493/503-stained area. (F) Hepatic triglyceride content in fasted mice. TG, triglycerides; scale bar = 200 µm; n = 7-8/group; data are expressed as means ± SD; * P <0.05.

Enhanced insulin signaling in the liver of Pten$^{+/-}$/Akt2$^{+/+}$ mice is partially dependent on AKT2

Insulin increases hepatic lipid content via inhibition of gluconeogenesis and stimulation of *de novo* lipogenesis [36-38]. Therefore, we examined whether diminished AKT signaling downstream of insulin might explain the reduced hepatic lipid content in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice and if this is dependent on AKT2.

Western blot analysis showed a more than 4.5-fold increase in phosphorylation of AKT at S473 (p-AKT S473) and T308 in the livers of fasted *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice (Fig. 3A). In *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice, p-AKT S473 was unchanged but p-AKT T308 was elevated 3-fold (Fig. 3A). Insulin-stimulated mice were analyzed to further assess activation of AKT. p-AKT S473 but not p-AKT T308 was 32% higher in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice than in control mice after insulin stimulation (Fig. 3A). As expected, the insulin-stimulated increase in p-AKT S473 and T308 in *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice was reduced by 72% and 41% compared to control mice, respectively (Fig. 3A). Analysis of the phosphorylation of the AKT targets GSK3β and FoxO1 showed an increase in p-GSK3β during fasting and in p-GSK3β and p-FoxO1 upon insulin stimulation in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice, in line with enhanced phosphorylation of AKT (Fig. 3B). *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice displayed increased p-GSK3β during fasting and increased p-FoxO1 upon insulin stimulation, showing that phosphorylation of GSK3β or FoxO1 in these mice is only partially dependent on AKT2 (Fig. 3B).

To further characterize hepatic insulin signaling, the expression levels of gluconeogenic and lipogenic genes were analyzed in fasted mice. Whilst FoxO1 phosphorylation did not change during fasting, expression of *Pepck* and *G6Pase* declined by more than 40% in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice (Fig. 3C). AKT2 was previously shown to upregulate expression of lipogenic genes in mice with hepatocyte-specific *Pten*-deficiency and *leptin*-deficient mice, but not in mice fed with normal chow or a specific high-fat diet [26,37].

Notably, the lipogenic genes *Srebp1-c* and its targets *Fas* and *Acc* were upregulated in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice (Fig. 3D). In *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice, expression of *Srebp1-c*, *Fas* and *Acc* was high but *Pparg* was downregulated compared to control mice (Fig. 3D). In order to clarify whether β-oxidation contributed to the reduced levels of hepatic lipids in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice, the expression levels of *Ppara* and *Pgc1a* were determined. There was no difference in the expression of *Ppara* or *Pgc1a* in fasted *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ compared to control mice (Fig. 3E).

Taken together, these data show that hepatic insulin signaling is elevated in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice and that this is mediated partially by AKT2. Moreover, the data suggest that extra-hepatic processes prevent the accumulation of lipids in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ liver.

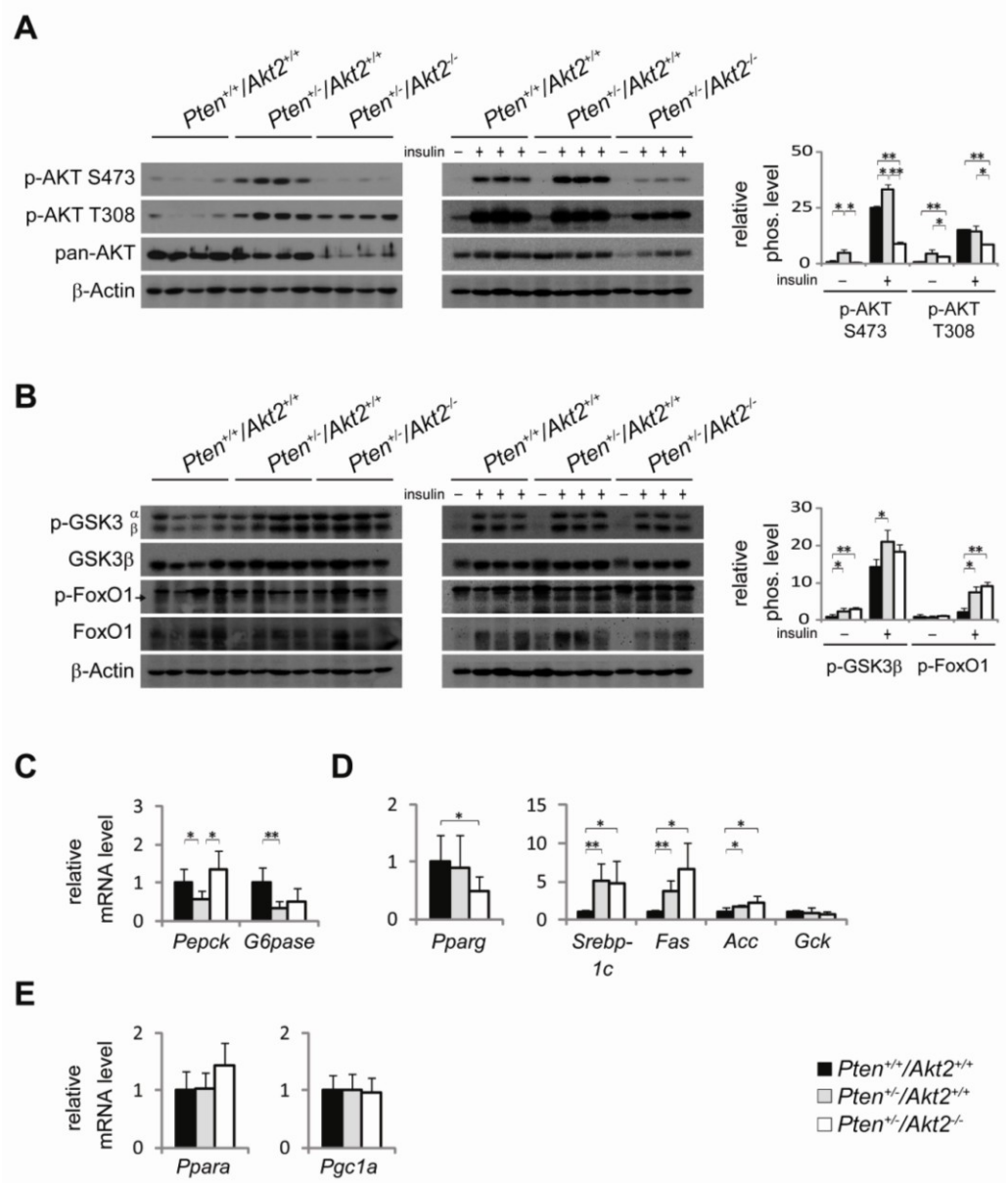

Figure 3. Enhanced AKT activation and upregulation of lipogenic genes in the liver of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice are partially dependent on AKT2. (A) Phosphorylation of AKT at S473 and T308 and pan-AKT protein levels in the liver of fasted and insulin stimulated mice. Densitometric quantification is shown. *n* = 3-4/group. (B) Phosphorylation and protein levels of GSK3β and FoxO1 in the liver of fasted and insulin stimulated mice. Densitometric quantification is shown. *n* = 3-4/group. (C, D, E) Relative mRNA levels of gluconeogenic genes (C), lipogenic genes (D) and genes involved in β-oxidation (E) in the liver of fasted mice. *n* = 6/group. Data are expressed as means ± SD; * P <0.05; ** P <0.01.

Increase in glycogen and enhanced AKT signaling in skeletal muscle of Pten$^{+/-}$/Akt2$^{+/+}$ mice depends on AKT2

Firstly, we characterized morphology of the pancreas and activation of AKT in the adipose tissue of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice, but no major changes were observed (Fig. S2, Fig. S3).

It was reported previously that the insulin sensitivity of skeletal muscle influences the accumulation of lipids in the liver through redistribution of ingested nutrients [12,39]. Furthermore, increased glucose uptake in skeletal muscle was found in *Pten*$^{+/-}$ mice after insulin stimulation [23]. Therefore, we examined whether an insulin response of skeletal muscle is involved in the reduction of hepatic lipids in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice.

The skeletal muscle of fasted *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice displayed increased triglyceride content, but no difference in glycogen content (Fig. 4A, B). However, glycogen content was increased in skeletal muscle of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice upon insulin stimulation, but was comparable to controls in *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice (Fig. 4C). These results indicate that skeletal muscle of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice displays an enhanced insulin response mediated by AKT2.

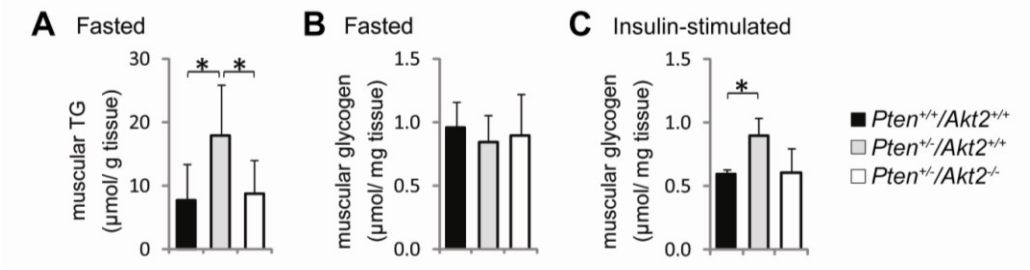

Figure 4. Triglyceride and glycogen contents are increased in skeletal muscle of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ but not *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice. (A) Triglyceride content in skeletal muscle of fasted mice. *n* = 6-8/group. (B, C) Glycogen content in skeletal muscle of fasted (B) and insulin-stimulated (C) mice. fasted *n* = 6-8/group; insulin-stimulated *n* = 3/group. TG, triglycerides; data are expressed as means ± SD; * P <0.05.

Next we analyzed basal and insulin-dependent activation of AKT and downstream targets in skeletal muscle by Western blotting. While AKT phosphorylation in fasted *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice was similar to control mice, fasted *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice displayed a reduction in p-AKT 473 by more than 70% (Fig. 5A). There were only minor differences in the phosphorylation of GSK3β and FoxO1 in fasted *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice (Fig. 5B). Notably, in insulin-stimulated *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice, p-AKT S473 but not p-AKT T308 was increased by 28% and p-GSK3β and p-FoxO1 were increased by more than 40% compared to control mice (Fig. 5A, B). In *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice, insulin-induced increase in p-AKT S473 and p-AKT T308 was abrogated (Fig. 5A). However, p-GSK3β and p-FoxO1 were induced by insulin in *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice similar to control mice, suggesting phosphorylation of GSK3β and FoxO1 is partially dependent on AKT2 in these mice (Fig. 5B).

While the expression of lipogenic genes in skeletal muscle of fasted *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice was at levels similar to controls, there was a trend towards reduced expression of *Ppara*, as well as a significant reduction of *Pgc1a* mRNA levels by more than 20%, suggesting diminished β-oxidation (Fig. 5C, D).

Taken together, our data reveal an enhanced insulin response of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ skeletal muscle that is mediated by AKT2. Hence, AKT2 in skeletal muscle may contribute to the reduction in hepatic lipids in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice and affect hepatic lipid accumulation in general.

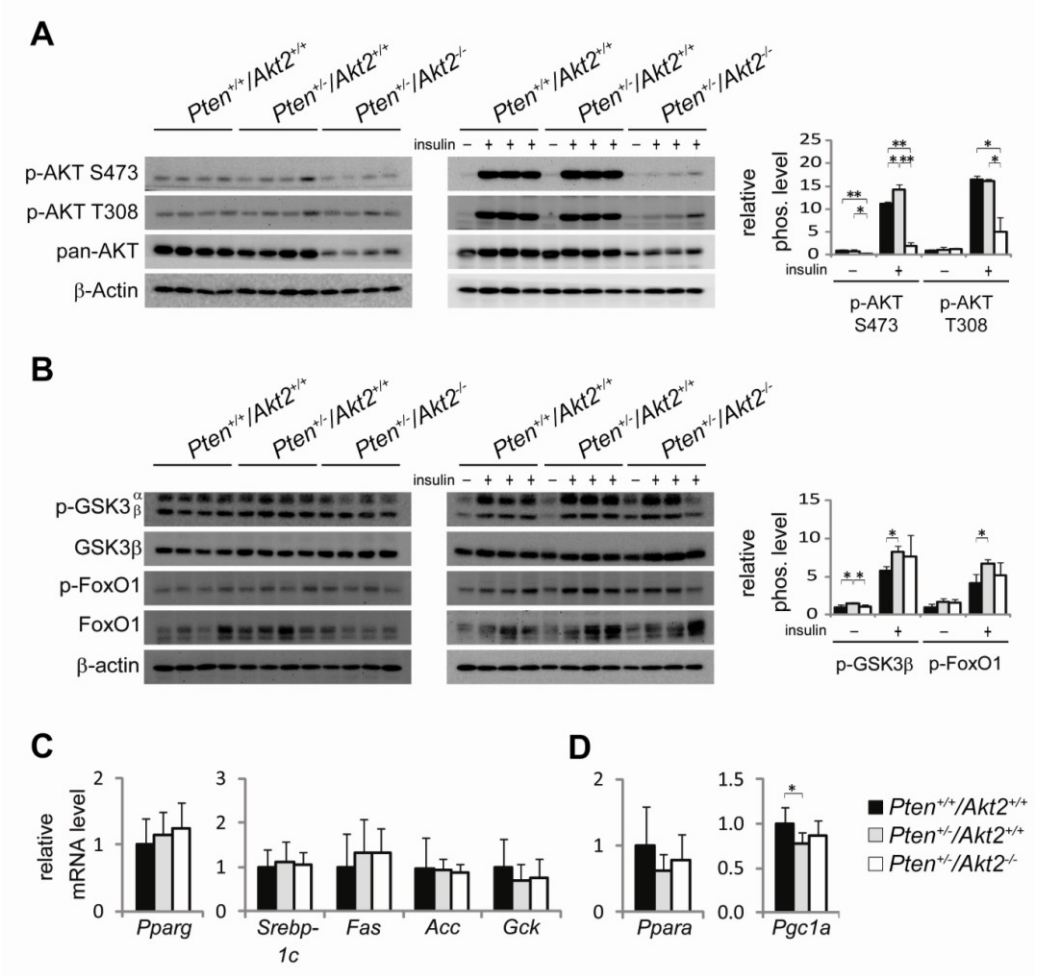

Figure 5. AKT signaling is enhanced in skeletal muscle of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice. (A) Phosphorylation of AKT at S473 and T308 and pan-AKT protein levels in skeletal muscle of fasted and insulin stimulated mice. Densitometric quantification is shown. *n* = 3-4/group. (B) Phosphorylation and protein levels of GSK3β and FoxO1 in skeletal muscle of fasted and insulin stimulated mice. Densitometric quantification is shown. *n* = 3-4/group. (C, D) Relative mRNA levels of lipogenic genes (C) and genes involved in β-oxidation (D) in skeletal muscle of fasted mice. *n* = 5-8/group. Data are expressed as means ± SD; * *P* <0.05; ** *P* <0.01.

Skeletal muscle-specific expression of AKT2 mutants affects hepatic lipid content

To analyze the effects of AKT2 in skeletal muscle on the accumulation of hepatic lipids and its possible contribution to reduced hepatic lipids in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice, constitutive active AKT2 (myr-AKT2) and dominant negative AKT2 (AKT2^{K180A}) were expressed specifically in skeletal muscle. Adeno-associated virus (AAV) 8 vectors expressing myr-AKT2 and AKT2^{K180A} with a GFP reporter gene were produced and skeletal muscle-specific expression was achieved by intraperitoneal injection of neonatal *Pten*$^{+/+}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice [31]. Mice expressing the different transgenes in skeletal muscle were designated as *Pten*$^{+/+}$/2A.GFP, *Pten*$^{+/+}$/myr-AKT2.2A.GFP, *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP, *Pten*$^{+/-}$/2A.GFP and *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP. The efficiency and specificity of transgene expression was validated by GFP staining of skeletal muscle and liver (Fig. 6A).

Mice expressing AKT2 mutants in skeletal muscle had body weights and fasted blood glucose concentrations similar to GFP-expressing control mice (data not shown). Glucose tolerance tests were performed to further assess glycemic control. *Pten*$^{+/+}$/myr-AKT2.2A.GFP and *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP showed glucose tolerance similar to the respective control mice. However, glucose tolerance of *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP mice was impaired as evidenced by an increase in the area under the glucose curve by 30% (Fig. 6B).

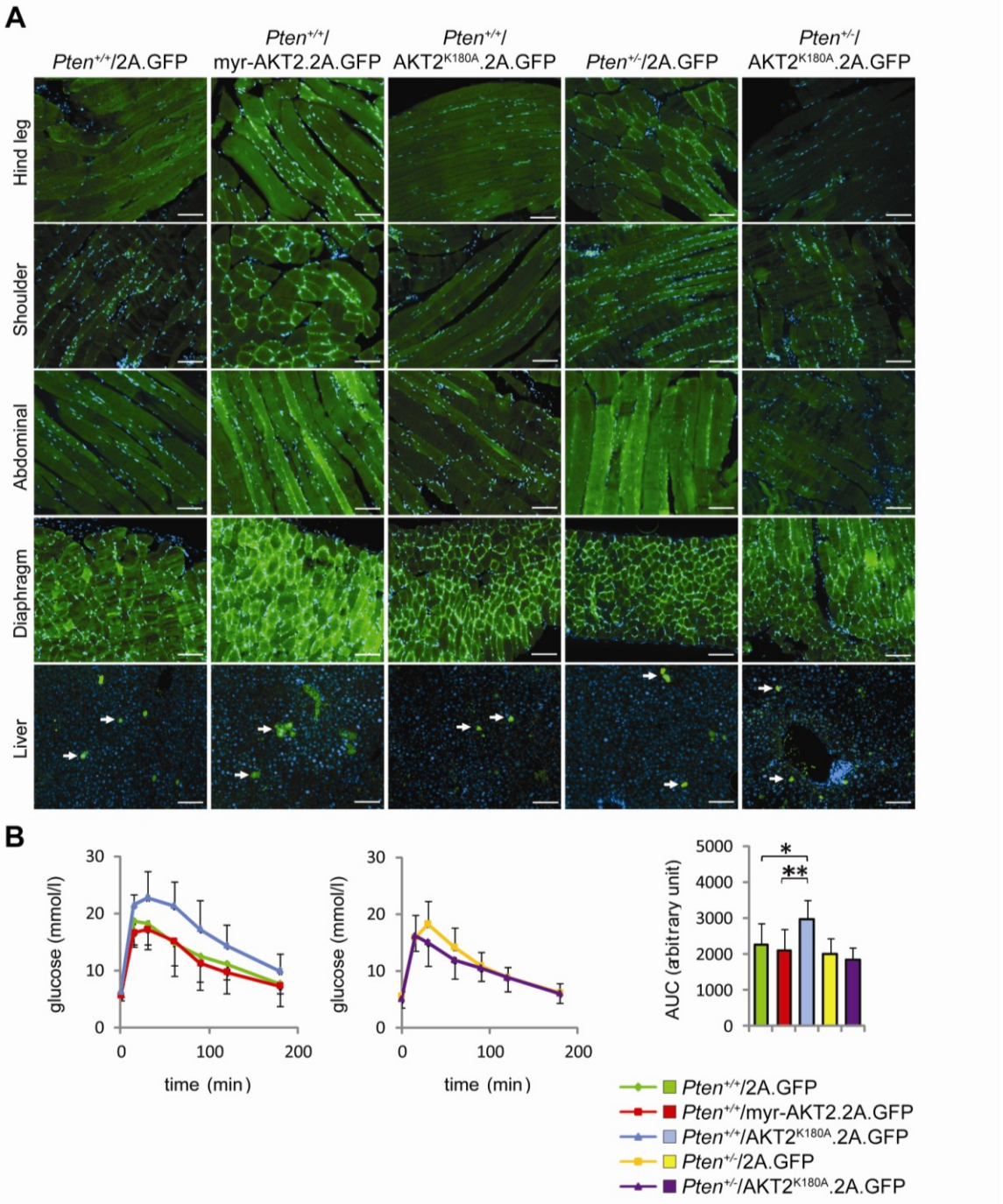

Figure 6. Skeletal muscle-specific expression of 2A.GFP, myr-AKT2.2A.GFP and AKT2^{K180A}.2A.GFP in *Pten*$^{+/+}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2* $^{+/+}$ mice. (A) Representative images of sections from indicated regions of skeletal muscle and liver stained for GFP (green) and DNA (blue). Arrows indicate GFP-positive hepatocytes. Scale bar = 100 µm. (B) Blood glucose concentrations in fasted mice after intraperitoneal administration of glucose were measured at the indicated time points. Time course of changes in blood glucose concentration are separated according to genotype for a better overview. Respective AUCs are shown. *n* = 7-12/group*;* AUC, area under the curve; data are expressed as means ± SD; * *P* <0.05, ** *P* <0.01.

Expression of AKT2 mutants in skeletal muscle did not alter liver weights (Fig. 7A). H&E-staining of liver sections revealed increased lipid accumulation in *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP mice (Fig. 7B). Notably, staining of liver sections with Oil Red O and BODIPY493/503 revealed less hepatic lipids in *Pten*$^{+/+}$/myr-AKT2.2A.GFP mice and an increase in *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP mice relative to *Pten*$^{+/+}$/2A.GFP controls (Fig. 7C, D). Moreover, *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP mice had a higher lipid content than *Pten*$^{+/-}$/2A.GFP mice (Fig. 7C, D). Importantly, these observations were confirmed by the quantification of BODIPY493/593-positive stained areas and triglyceride assays (Fig. 7E, F).

Taken together, the present data clearly show that enhanced activity of AKT2 in skeletal muscle is a key factor in the reduction of hepatic lipid content in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice. The significance of AKT2 activity in skeletal muscle on accumulation of hepatic lipids is further underlined by the effects observed in *Pten*$^{+/+}$*/Akt2*$^{+/+}$ mice expressing AKT2 mutants in the skeletal muscle. The higher hepatic lipid content in *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP compared to *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP mice, however, indicates the existence of further mechanism(s) influencing the accumulation of hepatic lipids in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice.

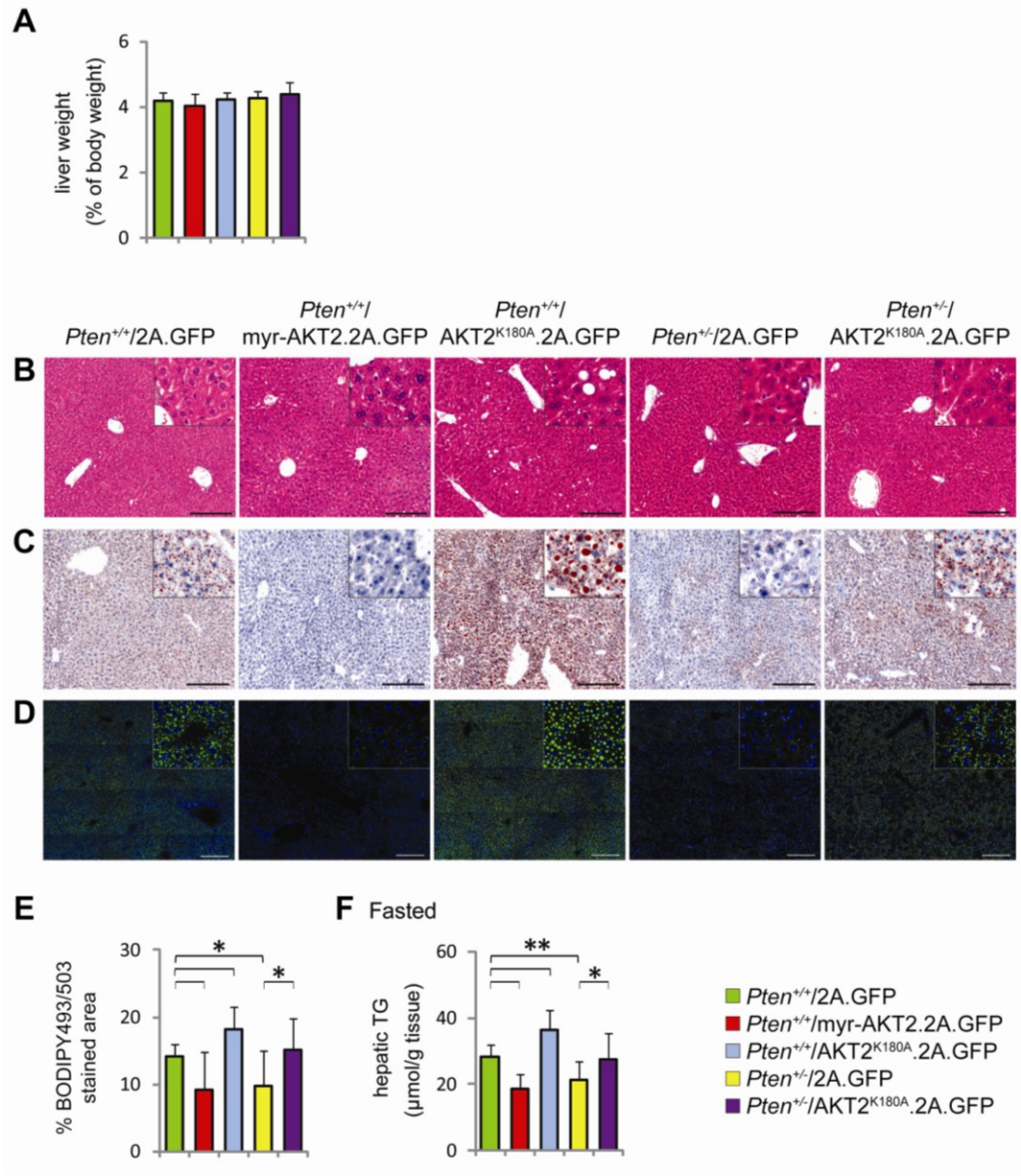

Figure 7. Skeletal muscle-specific expression of myr-AKT2 and AKT2^{K180A} affects hepatic lipid content. (A) Liver to body weight ratios. (B, C, D) Representative images of liver sections stained with H&E (B), for lipids with Oil Red O (C) and for lipids and DNA with BODPIY493/503 (green) and DAPI (blue), respectively (D). Image inlays show part of the depicted area at higher magnification. (E) Relative BODIPY493/503-stained areas. (F) Hepatic triglyceride content in fasted mice. TG, triglycerides; scale bar = 200 µm; n = 7-12/group; data are expressed as means ± SD; * P <0.05, ** P <0.01.

Discussion

The present study has characterized the contribution of peripheral insulin-sensitive tissues to lipid accumulation in liver upon loss of PTEN. Previous reports proposed that lipid accumulation in PTEN-deficient liver is driven by enhanced *de novo* lipogenesis due to hyperactivated AKT2 [24,25]. Indeed, deletion of AKT2 was found to inhibit the development of hepatic steatosis in mouse models with hepatocyte-specific *Pten*-deficiency, in *leptin*-deficient mice and in mice fed a high-fat diet [26,39]. However, the accumulation of lipids in the liver might not only depend on hepatic AKT2 activity but also on AKT2 activity in peripheral insulin-sensitive tissues via systemic interactions. To examine this, we used mice with a whole-body *Pten*-haplodeficiency, which in contrast to previous models have reduced PTEN levels in all tissues, including liver, pancreas, adipose tissue and skeletal muscle. $Pten^{+/-}/Akt2^{-/-}$ mice were also included in order to characterize the role of AKT2 signaling in the accumulation of hepatic lipids in this mouse model.

In contrast to hepatic steatosis in mice with hepatocyte-specific *Pten*-deficiency, we found that $Pten^{+/-}/Akt2^{+/+}$ mice have a significantly reduced hepatic lipid content, however, with similarly enhanced activation of hepatic AKT signaling [24,26]. $Pten^{+/-}/Akt2^{+/+}$ mice showed significantly increased activation of AKT and upregulation of lipogenic genes, but no differences in expression of genes involved in β-oxidation. The enhanced hepatic insulin signaling we observed indicates that extra-hepatic factors prevent accumulation of lipids in the livers of $Pten^{+/-}/Akt2^{+/+}$ mice. To define the factors reducing hepatic lipids in $Pten^{+/-}/Akt2^{+/+}$ mice, we characterized pancreatic morphology and insulin signaling in adipose tissue and skeletal muscle.

Several lines of evidence suggest that hepatic lipid content of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice might be affected by PTEN and AKT2 activity in pancreas and adipose tissue. Pancreas-specific deletion of *Pten* leads to hyperplastic pancreatic islets and ductal metaplasia and also improved metabolic control and elevated hepatic AKT signaling [40,41]. We found that the exo- and endocrine part of the pancreas of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice displayed overall normal morphology. Adipocyte-specific deletion of *Pten* was shown to result in hyperactivated AKT and improved glycemic control [42]. Enhanced energy expenditure in adipocytes was shown to reduce hepatic lipid content in mice [43]. In this study, AKT phosphorylation and expression of genes involved in energy expenditure in adipose tissue of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice was not changed. Thus, *Pten*-haplodeficiency in pancreas and adipose tissue might have only minor effects on hepatic lipid accumulation. Nevertheless, it merits further investigations, preferably using tissue-specific knockout mice, if PTEN and AKT2 in pancreas, adipose tissue and also other tissues have effects on hepatic metabolism that were not detected in this study.

Studies in humans have shown that insulin resistance of skeletal muscle promotes the development of NAFLD by redistribution of ingested carbohydrates towards hepatic *de novo* lipogenesis [12,39,44]. Moreover, hepatic lipid content was found to be reduced after insulin sensitivity of skeletal muscle was improved by physical exercise [12,39,44]. Another study showed that ectopic expression of constitutively active AKT1 in skeletal muscle of mice protected against diet-induced hepatic steatosis, by increasing β-oxidation in the liver [45]. Our results demonstrate an enhanced insulin response of skeletal muscle in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice as evidenced by increased glycogen content and AKT signaling upon insulin stimulation, which is mediated by AKT2. This data indicate that AKT2 in the skeletal muscle of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice is

a key factor in the reduction of hepatic lipid accumulation. Indeed, skeletal muscle-specific expression of AKT^{K180A} increased hepatic lipid content in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. Hence, these data support the hypothesis that an enhanced skeletal muscle insulin response mediated by AKT2 is the predominant factor preventing accumulation of lipids in the liver of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. The crucial role of skeletal muscular AKT2 in the accumulation of hepatic lipids is further underlined by the effects of skeletal muscle-specific expression of myr-AKT2 and $AKT2^{K180A}$ in *Pten*$^{+/+}$/*Akt2*$^{+/+}$ mice, which resulted in reduced and increased hepatic lipid content, respectively.

The expression of lipogenic genes in the liver of *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice was at similarly high levels as observed in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice, showing that the expression is only partially dependent on AKT2 in these mice. This is in line with previous findings showing that the regulation of lipogenic genes by AKT2 is context-dependent [24,26,37]. While in mice with hepatocyte-specific *Pten*-deficiency and *leptin*-deficiency the expression of lipogenic genes was found to be dependent on AKT2, the expression was not altered in *Akt2*-deficient mice fed with normal chow or a high-fat diet enriched in simple carbohydrates (Surwit diet) [24,26,37]. Activation of AKT2 in skeletal muscle of *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice is reduced; however, hepatic lipid content is as low as observed in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. This supports previous findings indicating that AKT2 is required for lipid accumulation in a hepatocyte-autonomous manner [37].

Despite the fact that hepatic *de novo* lipogenesis is a bona fide insulin response, NAFLD frequently occurs in insulin-resistant and diabetic patients. Recent studies have suggested that insulin resistance of skeletal muscle is a central factor in the development of NALFD [12,39,44,46]. In addition, a model of selective hepatic insulin resistance was proposed that

promotes NAFLD development. According to this model insulin fails to inhibit gluconeogenesis but still induces hepatic *de novo* lipogenesis in the liver [11]. Due to steady output of glucose from the liver insulin levels remain elevated, which further boosts hepatic *de novo* lipogenesis and accumulation of lipids [11]. The present study shows that hepatic lipid content is low in $Pten^{+/-}/Akt2^{+/+}$ mice despite increased activation of AKT2 and upregulation of lipogenic genes in the liver, and that an enhanced skeletal muscle insulin response reduces the accumulation of hepatic lipids. Hence, our data support the model that skeletal muscle insulin resistance is a central factor in the development of NAFLD. Thus, improving the insulin response in skeletal muscle by exercise and/or insulin sensitizer may be an effective option for treatment of NALFD.

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Supplementary figures

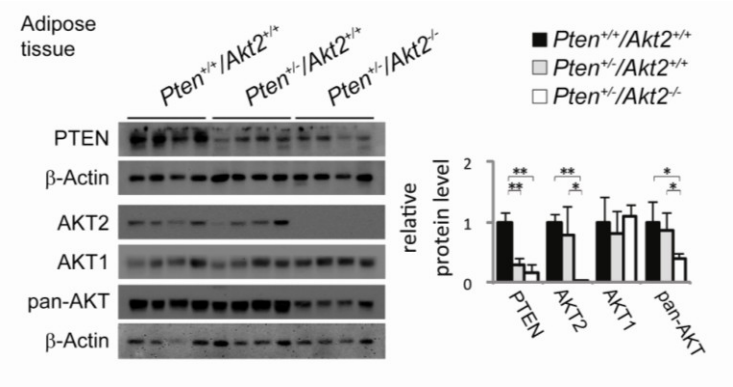

Figure S1. Validation of gene targeting in adipose tissue. Protein levels of PTEN, AKT2, AKT1 and pan-AKT in adipose tissue. Densitometric quantification is shown. n = 4/group; data are expressed as means ± SD; * P <0.05; ** P <0.01.

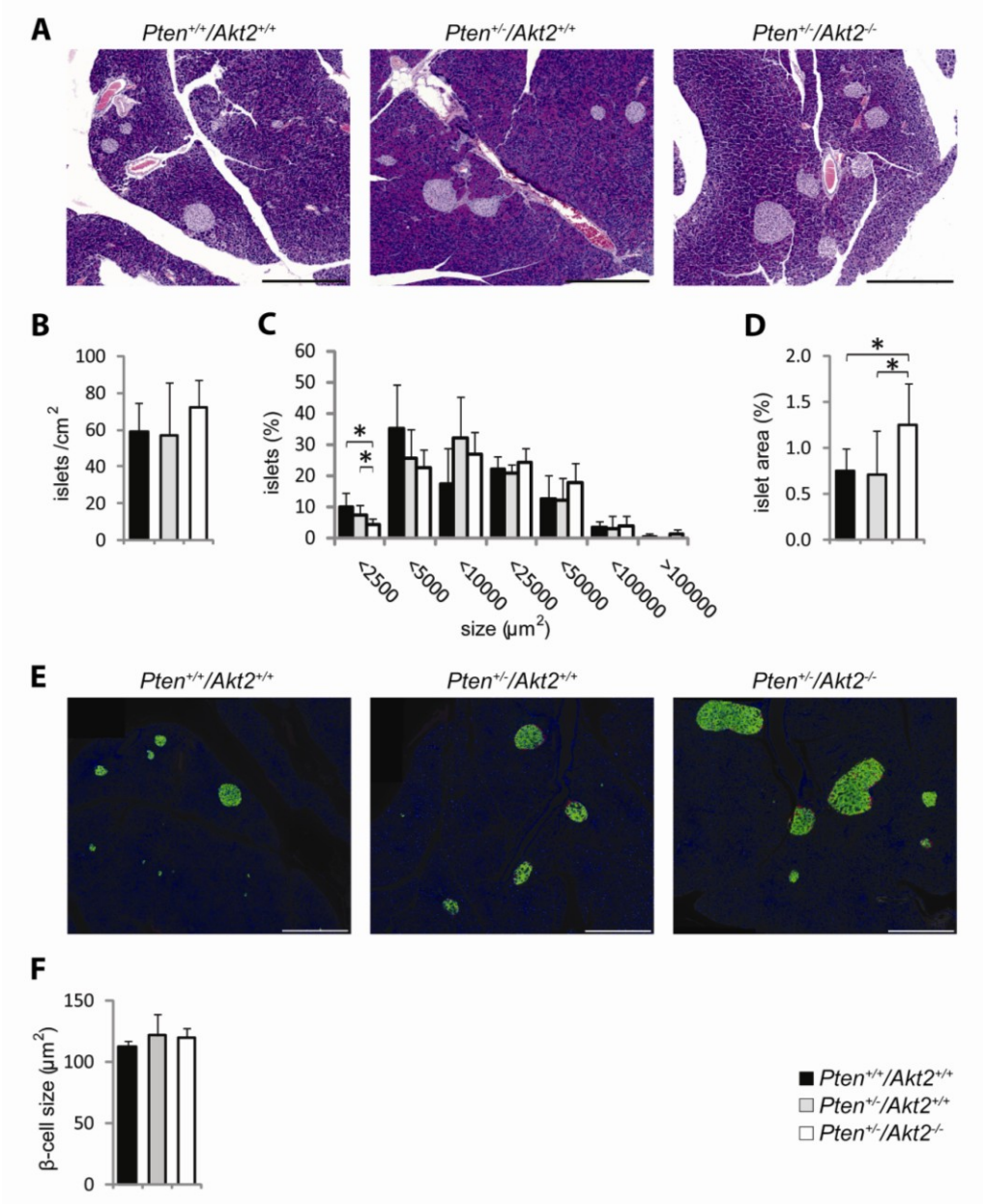

Figure S2. Overall normal pancreatic morphology in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice and increased β-cell mass in *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice. (A, B, C, D) Representative images of H&E-stained pancreatic sections (A) and the relative numbers of islets (B), size distribution of islets (C) and islet area (D). (E, F) Representative images of pancreatic sections stained for insulin (green), glucagon (red) and DNA (blue) (E), and the quantification of β-cell size (F). Scale bar = 500 μm; *n* = 5-8/group; data are expressed as means ± SD*; * P* <0.05.

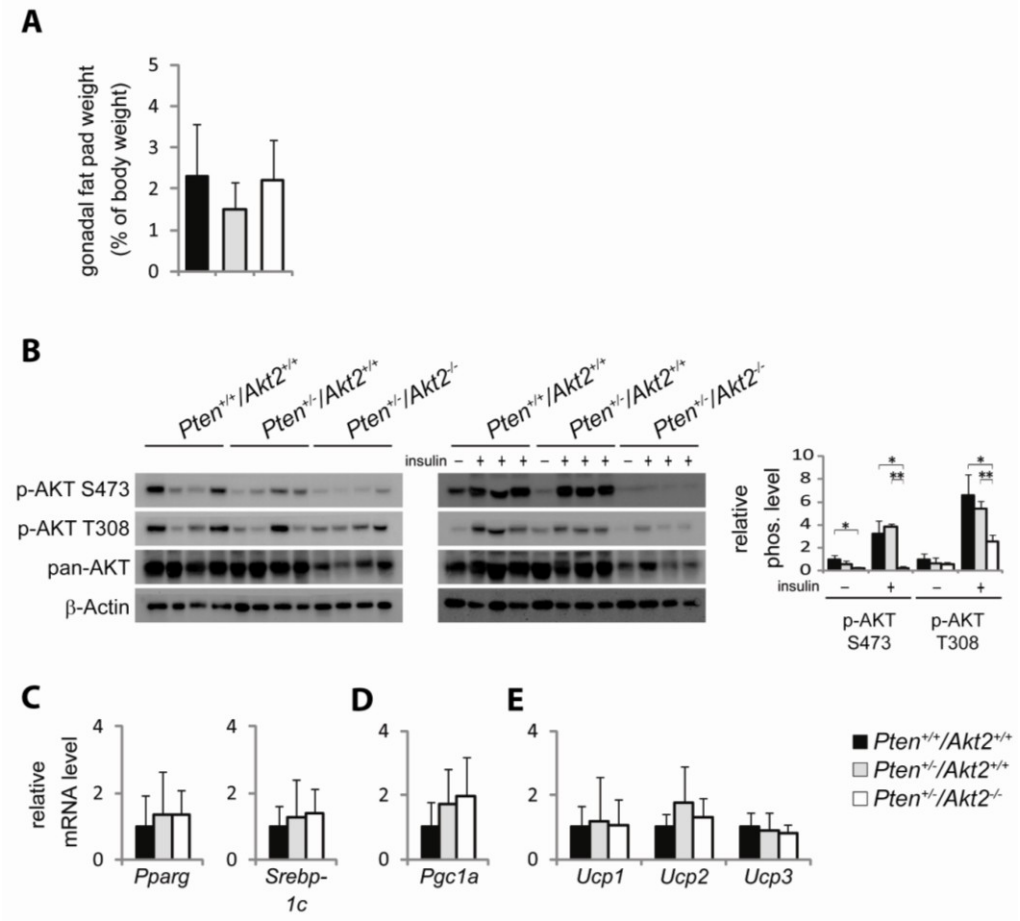

Figure S3. Insulin-induced AKT phosphorylation in adipose tissue is not affected in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ but is greatly diminished in *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice. (A) Gonadal fat pad to body weight ratios. *n* = 5-9/group (B) Phosphorylation of AKT at S473 and T308 and pan-AKT protein levels in adipose tissue of fasted and insulin stimulated mice. Densitometric quantification is shown. *n* = 3-4/group. (C, D, E) Relative mRNA levels of lipogenic genes (C), genes involved in β-oxidation (D) and genes implicated in energy expenditure (E) in adipose tissue of fasted mice. *n* = 5-8/group. Data are expressed as means ± SD; * P <0.05; ** P <0.01.

4. General discussion

Despite extensive efforts the molecular mechanisms of NAFLD development have not been fully elucidated. Recently, several components of the insulin signalling pathway such as PTEN, PKBβ and mTORC1 were shown to be involved in NAFLD development in a hepatocyte-intrinsic manner in mice (81, 82, 108, 109, 120, 122). However, the metabolic state of the liver does not merely depend on hepatic insulin response but also on peripheral insulin sensitive tissues via systemic interaction. The aim of this thesis was to characterize the effects of systemically perturbed PTEN/PKBβ signaling on accumulation of hepatic lipids. To examine this, we used mice with a whole-body *Pten*-haplodeficiency, which in contrast to previous models have reduced PTEN levels in all tissues, including liver, pancreas, adipose tissue and skeletal muscle. $Pten^{+/-}/Pkb\beta^{-/-}$ mice were also included in order to characterize the potential role of PKBβ signaling in the accumulation of hepatic lipids in this mouse model.

In this study we found that, in contrast to spontaneous development of hepatic steatosis in mice with liver-specific *Pten*-deficiency, $Pten^{+/-}/Pkb\beta^{+/+}$ mice have significantly reduced hepatic lipids. $Pten^{+/-}/Pkb\beta^{+/+}$ livers displayed enhanced activation of PKBβ and upregulation of lipogenic genes, indicating that accumulation of lipids in the liver is prevented by extra-hepatic factors. Analysis of peripheral tissues suggested that enhanced insulin signaling mediated by PKBβ in the skeletal muscle is a key factor in reduction of hepatic lipids. Indeed, skeletal muscle-specific expression of constitutively active PKBβ reduces hepatic lipids in $Pten^{+/+}/Pkb\beta^{+/+}$ mice and dominant negative PKBβ led to increased hepatic lipid content in both $Pten^{+/+}Pkb\beta^{+/+}$ and $Pten^{+/-}/Pkb\beta^{+/+}$ mice.

Deletion of PKBβ inhibits the development of hepatic steatosis in different mouse models of NAFLD (120, 122). Hence it was proposed that inhibition of PKBβ might be an effective target for treatment of NAFLD (122). However, systemic or liver-specific inhibition of PKBβ could deteriorate glycemic control (113, 118, 122). Our results show that activation of PKBβ in the skeletal muscle reduces hepatic lipid content, which can override lipogenic effects of hyperactivated PKBβ in the liver. These findings support the notion that skeletal muscle insulin resistance is a central factor in the development of NAFLD. Thus, improving the insulin response in skeletal muscle by physical exercise and/or skeletal muscle-specific insulin sensitizer may be an effective option for treatment of NALFD. Improved skeletal muscle insulin sensitivity would also be beneficial for glycemic control in obese and diabetic patients.

Today there is a high demand on novel therapies to counter the rising incidence of obesity, T2D and related complications such as cardiovascular disease, diabetic nephropathy and NAFLD (43). Lifestyle changes such as healthier diets and physical exercise improve insulin sensitivity and are very effective measures for prevention as well as treatment of obesity and T2D (11, 126-128). Healthier diet, physical exercise and weight loss reduce the risk of T2D progression in patients with impaired glucose tolerance by 30-60% (127, 129). In comparison, metformin, a widely used glucose lowering drug, reduces the risk of T2D progression by 31% (127). Importantly, lifestyle changes were found to have sustained effects (127). While preventive effects of glucose lowering drugs cease quickly after their withdrawal, a reduction in the risk of T2D progression maintains after lifestyle counseling stopped (36% relative risk reduction 3 years post-intervention) (127, 128). Lifestyle changes were also shown to be effective in NALFD treatment (34). For instance, 2 weeks of diet and exercise therapy in diabetic patients reduced

hepatic lipid content by 27% (34, 130). Weight loss by caloric restriction and physical exercise is therefore recommended as first line treatment of NAFLD (131). However, lifestyle changes have been proven difficult to achieve (11, 126).

Despite intensive clinical research, there is currently no standard pharmacological treatment for NAFLD and NASH. Treatment options of NAFLD and NASH by administration of bile acids, vitamin E or insulin sensitizer such as pioglitazone are under investigation (132-135). Vitamin D deficiency is increasingly recognized in NAFLD patients (136). In rats, vitamin D deficiency worsens NALFD and its supplementation by phototherapy has beneficial effects (136-138). Vitamin D administration may ameliorate NAFLD by several mechanisms such as increasing adiponectin level, reducing TNFα and IL-6 level and improving insulin sensitivity (136). Ongoing clinical trails are investigating the effects of vitamin D supplementation on liver histology in NAFLD and NASH patients (139, 140).

Studies in mice have demonstrated that inhibition of effectors and negative regulators of the insulin signaling pathway by genetic deletion can protect against genetically- and diet-induced obesity, insulin resistance and NAFLD (43). However, systemic modification of insulin signaling may have severe adverse effects. Negative regulators such as PTP1b and PTEN do not exclusively restrain insulin response but also regulate cell survival, differentiation and proliferation downstream of diverse growth factors and environmental stimuli (43). Global activation of the insulin signaling pathway by inhibition of PTEN is beneficial for metabolic control, but it also leads to cancer development (43). The PI3K/PKB/mTOR signaling pathway is inappropriately activated in many types of cancer, promoting growth and survival of cancer cells

(141). Thus, inhibitors targeting PI3K, PKB and mTOR are in clinical development and also successfully used in cancer therapy such as Everolimus and Temsirolimus in the treatment of metastatic renal cell carcinoma (43, 141, 142). Among several adverse effects, these inhibitors were found to impair metabolic control. Everolimus and Temsirolimus may cause hypercholesterolemia, hypertriglyceridemia and hyperglycemia with the need of subsequent lipid and glucose lowering treatments (43, 142). In contrast, tissue-specific inhibition of PI3K/PKB/mTOR signaling downstream of insulin can also improve metabolic control as shown in mice with adipocyte-specific deletion of *Insr* or *Raptor* (69, 87). Hence, the use of PI3K, PKB and/or mTOR inhibitors in a tissue-specifc manner might be beneficial for metabolic control. Tissue-specific targeting could be achieved by utilizing transmembrane carriers for selective cellular drug uptake, antibody-mediated drug delivery, metabolic activation of prodrugs or viral-mediated gene therapy (43). If adipocyte-specific inhibition of INSR and mTORC1 in humans would have effects similar to those observed in mice remains to be elucidated.

The positive effects on metabolic control observed in transgenic mouse models raise the hope for novel targeted therapies to treat obesity, T2D and related complications such as NAFLD. Understanding the mechanisms of context- and stimuli-specific function of potential targets as well as tissue-specific and long-term effects of modified activity will allow the development of therapies with minimized adverse effects tailored to individual demands (43).

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6. Appendix

6.1. PI3K/AKT, MAPK and AMPK signalling: protein kinases in glucose homeostasis.

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Abstract

New therapeutic approaches to counter the increasing prevalence of obesity and type 2 diabetes mellitus are in high demand. Deregulation of the phosphoinositide-3-kinase (PI3K)/v-akt murine thymoma viral oncogene homologue (AKT), mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) pathways, which are essential for glucose homeostasis, often results in obesity and diabetes. Thus, these pathways should be attractive therapeutic targets. However, with the exception of metformin, which is considered to function mainly by activating AMPK, no treatment for the metabolic syndrome based on targeting protein kinases has yet been developed. By contrast, therapies based on the inhibition of the PI3K/AKT and MAPK pathways are already successful in the treatment of diverse cancer types and inflammatory diseases. This contradiction prompted us to review the signal transduction mechanisms of PI3K/AKT, MAPK and AMPK and their roles in glucose homeostasis, and we also discuss current clinical implications.

Abstract taken from (43).

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PI3K/AKT, MAPK and AMPK signalling: protein kinases in glucose homeostasis

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New therapeutic approaches to counter the increasing prevalence of obesity and type 2 diabetes mellitus are in high demand. Deregulation of the phosphoinositide-3-kinase (PI3K)/v-akt murine thymoma viral oncogene homologue (AKT), mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) pathways, which are essential for glucose homeostasis, often results in obesity and diabetes. Thus, these pathways should be attractive therapeutic targets. However, with the exception of metformin, which is considered to function mainly by activating AMPK, no treatment for the metabolic syndrome based on targeting protein kinases has yet been developed. By contrast, therapies based on the inhibition of the PI3K/AKT and MAPK pathways are already successful in the treatment of diverse cancer types and inflammatory diseases. This contradiction prompted us to review the signal transduction mechanisms of PI3K/AKT, MAPK and AMPK and their roles in glucose homeostasis, and we also discuss current clinical implications.

Metabolic syndrome is generally defined as a cluster of risk factors for cardiovascular disease and type 2 diabetes mellitus (T2DM) including central obesity, arterial hypertension, dyslipidaemia and elevated fasting glucose (Ref. 1). Impaired glucose homeostasis, as observed in patients with metabolic syndrome, frequently progresses to overt T2DM, which in 2010 affected 344 million patients worldwide (Ref. 2). Hyperglycaemia in diabetic patients can lead to life-threatening complications such as coronary heart disease, stroke and nonalcoholic fatty liver disease (Refs 3, 4, 5).

Strict control of the level of circulating glucose within a narrow physiological range supplies sufficient energy for organs and avoids hyperglycaemia. Glucose homeostasis is largely maintained by the insulin–glucagon system, which compensates for physiological fluctuations in blood glucose caused by food intake and physical activity, or by stress conditions such as hypoxia and inflammation.

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Insulin and glucagon are released from β- and α-cells, respectively, in the endocrine part of the pancreas. Insulin lowers blood glucose by stimulating glucose uptake and storage (glycogen synthesis and lipogenesis) in skeletal muscle and adipose tissue. In the liver, insulin blocks the release and neogenesis of glucose and stimulates glucose storage. In addition, insulin stimulates protein synthesis, regulates mitochondrial biogenesis and blocks autophagy. Glucagon antagonises the action of insulin, mostly in the liver, where it stimulates gluconeogenesis and thereby increases blood glucose level. The secretion of insulin and glucagon is regulated in a reciprocal manner, which avoids glycaemic volatility because of their opposing effects. It was proposed that the glucose-induced secretion of insulin inhibits glucagon secretion from α-cells in a paracrine manner (Ref. 6). Furthermore, incretin hormones [e.g. glucagon-like peptide 1 (GLP-1)] secreted postprandially by the gut potentiate glucose-mediated insulin secretion and block glucagon secretion (Ref. 7). In addition, physiological conditions such as low intracellular energy level and cellular stress affect whole-body glucose homeostasis by interfering with insulin action.

Signal transduction from a stimulus to the regulation of cellular processes, including those involved in glucose homeostasis, is primarily dependent on protein kinase signalling. On activation, protein kinases determine the output of metabolic processes by transcriptional and post-translational regulation of rate-limiting enzymes, such as glycogen synthase 1 (GYS1) and fatty acid synthase (FASN, FAS). The insulin receptor (INSR, IR) activates various downstream pathways that control energy homeostasis, including phosphoinositide-3-kinase (PI3K)/v-akt murine thymoma viral oncogene homologue [AKT, also known as protein kinase B (PKB)] and the mitogen-activated protein kinase 3/1 (MAPK3/1, ERK1/2). Whereas the PI3K/AKT pathway is considered to be the major effector of metabolic insulin action, insulin-independent kinases also contribute to metabolic control. AMP-activated protein kinase (AMPK) is mostly activated by low intracellular energy levels and inhibits anabolic processes, stimulates energy-producing catabolic processes and lowers blood glucose level. Because correct functioning of the PI3K/AKT, MAPK and AMPK pathways is essential for proper metabolic control and their dysfunction often leads to impaired glucose homeostasis, these pathways are attractive therapeutic targets (Refs 8, 9, 10). However, PI3K/AKT, MAPK and AMPK are also involved in several other fundamental cellular processes, including cell proliferation and survival, and thus global therapeutic modification of their activities could induce severe side effects.

Today, specific kinase inhibitors are used successfully for immunosuppression and in the treatment of inflammatory disease and diverse cancer types. However, because proper activation of the PI3K/AKT pathway is required for insulin action, kinase inhibitors targeting PI3K/AKT and downstream effectors might impair metabolic control. Even though inappropriate activation of MAPKs, especially of c-Jun N-terminal kinase (MAPK8, JNK), is considered to have a critical role in acquired insulin resistance, no therapies based on MAPKs are available so far. The only drug targeting protein kinase activity that is widely used today in the treatment of insulin resistance and diabetes is metformin, which is thought to operate mainly by activating AMPK. Although our understanding of the role of protein kinases in the regulation of glucose homeostasis has increased significantly during the past decade, only limited translation into therapies against the metabolic syndrome has occurred. The purpose of this present review is to summarise the signal transduction mechanisms involving PI3K/AKT, MAPK and AMPK with respect to their role in glucose homeostasis and to discuss current clinical implications.

The PI3K–AKT signalling pathway is the major effector of metabolic insulin action

Insulin is an indispensable regulator of glucose homeostasis, and T2DM is characterised by postreceptor insulin resistance combined with β-cell failure. Insulin signalling is initiated by the binding of insulin to the extracellular α-subunits of the heterotetrameric IR. This interaction induces conformational changes and facilitates autophosphorylation of tyrosine residues on the intracellular part of membrane-spanning β-subunits. These phosphotyrosines then attract a family of adaptor molecules, the insulin receptor substrates (IRSs). On interaction with the IR, IRS proteins themselves are tyrosine phosphorylated, which is partially mediated by

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the tyrosine kinase activity of the IR and also by other kinases. Once phosphorylated, IRS proteins attract downstream signalling molecules, thereby linking the activated IR to the various downstream signalling pathways (Ref. 11).

Molecular mechanism of the PI3K/AKT signalling pathway downstream of insulin

The PI3K/AKT pathway is required for insulin-dependent regulation of systemic and cellular metabolism (Ref. 8). Besides insulin, many other growth factors, cytokines and environmental stresses can activate PI3K/AKT, mainly in the regulation of cell proliferation, motility, differentiation and survival (Ref. 12). Thus, PI3K/AKT action is highly context dependent, which is at least partially mediated by the recruitment of different isoforms of PI3K (including p85α, p110α, p110β) and AKT (AKT1, AKT2, AKT3) downstream of individual stimuli (Refs 13, 14, 15). The AKT isoforms are encoded by individual genes located on different chromosomes, share approximately 80% identity in their amino acid sequences and form the same protein structure, including an N-terminal pleckstrin homology (PH), a catalytic domain and a C-terminal regulatory domain (Ref. 16). Among the AKT isoforms, AKT2 is considered to be the major isoform required for metabolic insulin action. Although intensively investigated, the exact molecular mechanisms underlying isoform and context specificity are still not fully elucidated. Here we focus on the function of the PI3K/AKT pathway downstream of IR and IRS proteins and its role in glucose homeostasis.

The PI3K/AKT pathway is activated downstream of the IR by binding an SH2 domain within the regulatory subunit of PI3K (p85) to phosphotyrosines in IRS1/2. This leads to recruitment and activation of the catalytic subunit of PI3K (p110). Once activated, PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) at the plasma membrane. AKT binds through its PH domain to PIP3, which facilitates activation of AKT by upstream kinases. Initially, 3-phosphoinositide-dependent protein kinase-1 (PDPK1, PDK1) induces about 10% of kinase activity by phosphorylating Thr308 in the catalytic domain of AKT. Subsequently, mammalian target of rapamycin complex 2 (mTORC2), DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated kinase (ATM) induce full kinase activity of AKT by phosphorylating Ser473 in the regulatory domain. Although DNA-PK can phosphorylate AKT at Ser473 on insulin stimulation in vitro, it is thought to activate AKT in vivo mainly following stress such as DNA damage (Refs 17, 18, 19). mTORC2 is considered to be the predominant AKT Ser473 kinase downstream of insulin and growth factor stimuli (Ref. 18). On activation, AKT is released from the plasma membrane and translocates to cellular compartments, such as the cytoplasm, mitochondria and nucleus, where it phosphorylates its many substrates. Substrates implicated in the regulation of cellular metabolism include glycogen synthase kinase 3β (GSK3β), forkhead box protein O1 (FOXO1) and AKT substrate 160 (TBC1D4, AS160), which regulate glycogen synthesis, gluconeogenesis and glucose uptake, respectively. AKT also activates mTORC1 by inhibiting tuberous sclerosis complex 1/2 (TSC1/2). Activated mTORC1 upregulates mitochondrial biogenesis, inhibits autophagy and induces protein synthesis by regulation of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), unc-51-like kinase 1 (ULK1), and ribosomal protein S6 kinase (S6K) and eIF4E-binding protein 1 (4E-BP1), respectively. PDK1 also activates isoforms of protein kinase C (PKCλ/ζ), which are required for Glut4-dependent regulation of glucose uptake. Moreover, AKT and PKCλ/ζ control de novo lipogenesis by regulating lipogenic genes, such as sterol regulatory element-binding transcription factor 1 (SREBF1, SREBP1c) and peroxisome proliferator-activated receptor γ (PPARγ) (Refs 20, 21). The mechanisms by which AKT and PKCλ/ζ regulate lipogenic genes are not yet completely understood.

The insulin–PI3K/AKT pathway is negatively regulated at different levels. Phosphatases, including protein tyrosine phosphatase nonreceptor type 1 (PTPN1, PTP1B), phosphatase and tensin homologue (PTEN) and protein phosphatase 2A (PP2A), dephosphorylate and thereby inhibit IR, IRS1/2, PIP3 and AKT, respectively. AKT activity can also be inhibited by binding partners, such as thioesterase superfamily member 4 (THEM4, CTMP) and tribbles homologue 3 Drosophila

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(TRIB3) (Refs 22, 23). Whereas the function of most AKT-binding partners in glucose homeostasis remains to be elucidated, TRIB3 was shown to inhibit insulin signalling (Ref. 23). Furthermore, negative-feedback loops are implemented in the PI3K/AKT pathway that downregulate insulin signalling. GSK3β, mTORC1 and S6K can phosphorylate IRS on serine residues, which can lead to their ubiquitylation and proteolytic breakdown (reviewed in Refs 12, 24, 25) (Fig. 1).

Genetic alterations in components of the insulin signalling pathway can impair or improve metabolic control

Many studies have been carried out in mice and humans and have been pivotal in defining the molecular events underlying insulin signalling. Most patients develop insulin resistance and T2DM as a result of polygenetic predisposition in combination with overnutrition and obesity (acquired insulin resistance). Monogenetic defects causing diabetes account for only 1–5% of cases and have been found in loci encoding elements of the insulin signalling pathway, transcription factors and rate-limiting enzymes of glucose metabolism (e.g. hepatocyte nuclear factor 4α and glucokinase) and also in mitochondrial genes (Ref. 26). Interestingly, both enhancement of insulin signalling by deletion of negative regulators and specific interference with its action by deleting targets normally activated by insulin can improve metabolic control and protect against diabetes in mice.

From IR to AKT: genetic mutations and their effects on insulin sensitivity in humans and mice

Patients with loss-of-function mutations in IR are severely insulin resistant and display signs of hyperglycaemia and hyperinsulinaemia, thus indicating that IR is essential for insulin action (Refs 27, 28, 29). Experiments in vitro have confirmed that amino acid substitutions in the tyrosine kinase domain of IR found in patients, such as glycine (G) to valine (V) at position 996 (G996V) and Q1131R, indeed block insulin signalling, as shown by markedly reduced IR tyrosine kinase activity and diminished phosphorylation of IRS1/2 (Refs 28, 30).

Of the postreceptor gene mutations in the insulin signalling cascade, only a few were found to cause severe insulin resistance in humans. Several variants of *IRS1* and *IRS2* have been identified in patients with insulin resistance. Two IRS1 variants, a common (G972R) and a rare (T608R) polymorphism, were associated with reduced insulin sensitivity in obese men and severe insulin resistance, respectively (Refs 31, 32). Both polymorphisms are located in regions implicated in PI3K binding and abolished insulin-stimulated PI3K activity in cell culture models (Refs 32, 33). By contrast, variants of *IRS2* were not associated with insulin resistance, and their biochemical properties were not characterised (Refs 34, 35). Of the known polymorphisms in p85α and p110β subunits of PI3K, only an R409Q amino acid substitution in p85α was shown to compromise insulin-stimulated PI3K activity (Refs 36, 37). Remarkably, a mutation identified in *AKT2* resulting in an R274H amino acid substitution in the kinase domain was associated with autosomal dominant inherited severe insulin resistance. AKT2 R274H has greatly reduced kinase activity and acts in a dominant-negative manner in that its overexpression blocks the inhibition of FOXA2 in HepG2 cells and impairs adipocyte differentiation in vitro (Ref. 38).

Findings in transgenic mice complement the above observations. Mice deficient in the IR develop severe hyperglycaemia within hours after birth and die within days as a result of severe ketoacidosis (Refs 39, 40). IRS1-deficient mice have peripheral insulin resistance, but show only slight hyperglycaemia because of compensatory hyperinsulinaemia (Refs 41, 42). A more severe metabolic phenotype was observed in mice deficient in IRS2. These animals are also insulin resistant, but show hyperglycaemia as a result of impaired adaptation of β-cell mass (Ref. 43). By contrast, specific loss of elements of insulin signalling can also improve metabolic control. It was shown that mice with an adipose-tissue-specific deletion of *Ir* are protected against obesity and obesity-related insulin resistance (Ref. 44). Whereas p85α R409Q is associated with reduced insulin sensitivity in humans, loss of p85α by mutation of the corresponding gene *Pik3r1* (which encodes p85α, p55α and p50α) resulted in improved glucose tolerance and hypoglycaemia in mice (Refs 45, 46). It was suggested that the loss of p85α is compensated by p50α, which generated an increase in PIP3 on insulin stimulation (Ref. 45). However, there

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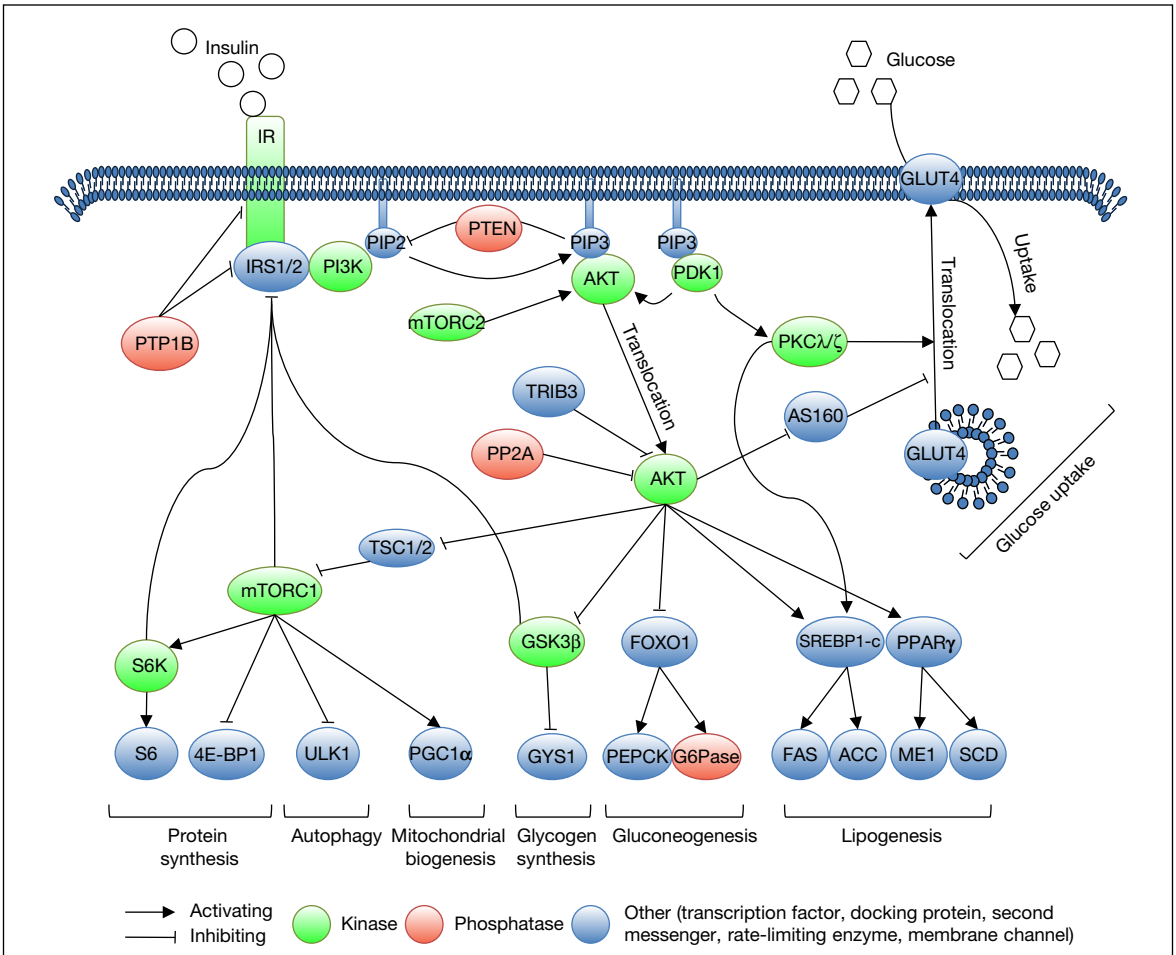

Simplified view of insulin-stimulated PI3K/AKT signalling and its substrates involved in cellular metabolism

Figure 1. Simplified view of insulin-stimulated PI3K/AKT signalling and its substrates involved in cellular metabolism. A detailed description is given in the text. Abbreviations: ACACA, ACC, acetyl-CoA carboxylase; AKT, v-akt murine thymoma viral oncogene homologue 1; 4E-BP1, eIF4E-binding protein 1; FOXO1, forkhead box O1; G6Pase, glucose-6-phosphatase; GSK3β, glycogen synthase kinase 3β; GYS1, glycogen synthase; INSR, IR, insulin receptor; IRS1/2, insulin receptor substrates 1/2; ME1, malic enzyme 1; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mTOR complex 2; PDPK1, PDK1, 3-phosphoinositide-dependent protein kinase-1; PGC1α, peroxisome proliferator-activated receptor gamma, coactivator 1α; PI3K, phosphoinositide-3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PKC1, PEPCK, phosphoenolpyruvate carboxykinase 1; PKCλ/ζ, protein kinase Cλ/ζ; PPARγ, peroxisome proliferator-activated receptor g; PP2A, protein phosphatase 2A; PTPN1, PTP1b, protein tyrosine phosphatase, non-receptor type 1; RPS6, S6, ribosomal protein S6; SCD, stearoyl-CoA desaturase; S6K, ribosomal protein S6 kinase; SLC2A4, GLUT4, solute carrier family 2; SREBF1, SREBP1-c, sterol regulatory element binding transcription factor 1; TBC1D4, AS160, AKT substrate 160; TRIB3, tribbles homologue 3; TSC1/2, tuberous sclerosis complex 1/2; ULK1, unc-51-like kinase 1.

may be further compensatory mechanisms, given that mice lacking all three isoforms of *Pik3r1* are also hypoglycaemic (Ref. 46). These studies demonstrate that ablation of proteins can have effects different from loss-of-function mutations and also from inhibitor treatments in which the

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inoperative protein remains present. For detailed descriptions of these mouse models, the reader is referred to another review (Ref. 47).

As described above, loss-of-function mutations in genes of the insulin signalling pathway mostly reduce insulin sensitivity to varying degrees. These findings support the notion that these genes are required for insulin action and are the basis of our understanding of the molecular mechanisms underlying insulin signalling and the development of diabetes. Thus, at first sight, it appears desirable to enhance insulin signalling in order to counteract the development of diabetes. However, the observation that adipose-tissue-specific IR deficiency can improve metabolic control and protect against obesity suggests an interesting alternative.

Diverse effects on glucose homeostasis are observed after deletion of individual AKT isoforms and downstream protein kinases in mice

The mechanisms of insulin signalling at the level of and downstream of, AKT isoforms have been studied extensively in transgenic mouse models. AKT1 and AKT2 are ubiquitously expressed, with high levels in classical insulin target tissues such as the liver, skeletal muscle and adipose tissue (Refs 48, 49). By contrast, the expression of AKT3 appears more restricted and is found mainly in the brain, the testis, adipose tissue and pancreatic islets (Refs 48, 49). As in the case of humans, mice lacking AKT2 are insulin resistant, hyperglycaemic and hyperinsulineamic (Refs 8, 48, 50). Deficiency in AKT3 does not result in metabolic aberrations. However, somewhat conflicting results have been obtained with mice deficient in AKT1. Two studies reported no role for AKT1; however, a third study described higher insulin sensitivity and improved metabolic control (Refs 48, 51, 52). The molecular mechanisms underlying this improved insulin sensitivity in AKT1-deficient mice have not been defined. Although highly similar in structure, loss of individual AKT isoforms results in distinct phenotypes, indicating that AKT isoforms exert nonredundant functions. This can be partially explained by divergent expression patterns, but we are far from understanding the molecular mechanisms underlying specificity (Ref. 15).

The results obtained in mouse models indicate that individual downstream effectors of AKT exert distinct and tissue-specific functions. GSK3β inhibits glycogen synthesis by phosphorylating GYS1 and is negatively regulated by AKT. Accordingly, mice with specific deletion of *Gsk3b* in skeletal muscle but not in the liver showed improved glucose tolerance owing to enhanced GYS1 activity and glycogen deposition (Ref. 53). Additionally, it was shown that mice with a pancreatic β-cell-specific deletion of *Gsk3b* display increased β-cell mass and improved glucose tolerance and are protected against genetically and diet-induced diabetes. This increase in β-cell mass might occur as a result of loss of GSK3β-mediated feedback inhibition of insulin signalling, which is known to increase β-cell proliferation (Refs 54, 55).

mTORC1 and its downstream target S6K are indirectly activated by AKT2, and their roles have also been studied in mice. Activation of mTORC1 in β-cells by deletion of *Tsc1* or *Tsc2* increased cell size, proliferation and insulin production. Thus, β-cell-specific activation of mTORC1 improved glucose-stimulated insulin secretion and glucose tolerance in mice (Refs 56, 57). Conversely, mice with a whole-body S6K deficiency showed reduced β-cell mass and hypoinsulinaemia (Ref. 58). Ablation of mTORC1 activity in skeletal muscle in mice by deletion of *Raptor* reduced oxidative capacity by the downregulation of genes involved in mitochondrial biogenesis. Moreover, the glycogen content of the muscle in these mice was increased, most likely because of enhanced inhibition of GSK3β by hyperactivated AKT. As a result, these mice suffered from progressive muscle dystrophy and were glucose intolerant (Ref. 59). Interestingly, mice with an adipocyte-specific mTORC1 deficiency as well as those with a whole-body S6K deficiency were protected against diet-induced obesity and insulin resistance. The authors proposed that the protective effects are based on increased energy expenditure and enhanced insulin signalling, which are probably due to loss of negative feedback regulation in adipose tissue (Refs 60, 61). Recently, it was shown that mice with liver-specific activation of mTORC1 by deletion of *Tsc1* are glucose intolerant but, are protected against diet-induced hepatic steatosis. The authors also showed that inhibition of mTOR by rapamycin does not reduce hepatic lipid accumulation in mice fed a high-fat diet. Thus, it

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was concluded that mTORC1 is not required and not sufficient to increase hepatic lipids, but rather protects against diet-induced hepatic steatosis by enhancing fat utilisation and gluconeogenesis in the liver (Ref. 62) (Table 1).

These findings show that not only AKT isoforms but also their downstream effectors perform distinct functions in the regulation of glucose homeostasis. Moreover, the impact on metabolic control of modulating the activity of downstream components in the insulin signalling cascade largely depends on the targeted tissue, as demonstrated in the case of mTORC1 and S6K. Thus, the development of techniques for tissue-specific, but not systemic, targeting of downstream components could allow further adaption of current therapies to individual demands, such as improving β-cell function, reducing hepatic lipid content and restoring insulin response in skeletal muscle.

Improved glucose homeostasis in mice lacking negative regulators of PI3K/AKT

As mentioned above, phosphatases such as PTP1B and PTEN antagonise insulin signalling. PTP1B downregulates insulin-stimulated PI3K/AKT signalling by dephosphorylating IR and IRS1/2 in a more specific manner than PTEN, which inhibits PI3K/AKT signalling by dephosphorylating PIP3. Because several other growth factors, such as EGF and PDGF, can also increase levels of PIP3 by stimulating PI3K, PTEN appears to be a critical antagonist of all PI3K-dependent AKT stimuli. Notably, both PTP1B deficiency and *Pten* hemizygosity result in improved glucose tolerance and insulin sensitivity in mice (Refs 63, 64). Similar phenotypes were found in mice with tissue-specific PTP1B deficiency in muscle or liver, and PTEN deficiency in muscle, adipose tissue or liver (Refs 65, 66, 67, 68, 69, 70). Furthermore, it was shown that mice with whole-body and muscle-specific PTP1B deficiency, and mice lacking PTEN in muscle and pancreas, are protected against diet-induced insulin resistance (Refs 63, 65, 67, 71). In contrast to PTP1B-deficient mice, mice with *Pten* hemizygosity and mice with PTEN deficiency in hepatocytes develop tumours in various organs or progressive hepatic steatosis with the development of liver cancer, respectively (Refs 69, 70, 72) (Table 2). These phenotypes indicate that PTEN is required to control growth-factor-stimulated PI3K/AKT signalling. Moreover, PTEN was shown to have a phosphatase-independent tumour-suppressive function in the nucleus, which might also have a role in tumour development in mice (Ref. 73).

Recent evidence suggests that the targeting of negative regulators further downstream, such as TRIB3, might enhance insulin signalling without global activation of the PI3K/AKT pathway. Whereas mice with whole-body TRIB3 deficiency showed no alterations in metabolic control under normal conditions, TRIB3 was shown to be upregulated in the liver of diabetic mice and hepatic overexpression of TRIB3 impaired glucose tolerance (Refs 23, 74, 75). Because TRIB3 seems to be dispensable under normal conditions, but seems to contribute to obesity-induced insulin resistance, it might represent an attractive therapeutic target.

As underlined by the complex phenotype of PTEN-deficient mice, the inhibition of negative regulators can lead to global activation of PI3K/AKT with severe side effects such as hepatic steatosis and cancer. Thus, the safe targeting of negative regulators of insulin signalling may be out of reach until the regulation of context-specific stimulation is understood. The targeting of negative regulators further downstream, such as TRIB3, could be more specific and have improved side-effect profiles.

mTOR inhibitors in clinical use and how they affect glucose homeostasis

Although results from the studies described above show that interfering with PI3K/AKT/mTOR signalling mostly leads to insulin resistance, its inhibition is an attractive treatment option for various other diseases. Inhibition of PI3K/AKT/mTOR signalling should be considered especially in cancer therapy, because inappropriate activation of this pathway is frequently observed in many tumour types. Indeed, the mTOR inhibitors temsirolimus and everolimus have been approved for the treatment of metastatic renal cell carcinoma (mRCC) and improve overall or progression-free survival (Refs 76, 77). Current trials explore the efficiency of mTOR inhibitors when used in combination with other therapies, including small-molecule tyrosine kinase inhibitors or VEGF-directed antibodies (Ref. 78). In addition to mRCC, an increasing number of clinical trials study the

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Table 1. Overview of mouse models for AKT isoforms and downstream targets

Gene	Deleted in	Insulin sensitivity	Glucose tolerance	Further characteristics	Refs
Akt1	Whole body	+	+	Reduced body size, increased neonatal mortality	48, 49
Akt2	Whole body	−	−	Diabetes-like phenotype with compensatory increase in pancreatic β-cell mass, protected against genetic- and diet-induced hepatic steatosis	8, 20, 48, 50, 159
	Hepatocytes	NR	NR	Protected against genetic- and diet-induced hepatic steatosis	20
Akt3	Whole body	UC	UC	Impaired postnatal brain development, no obvious metabolic phenotype	48, 160
Gsk3a	Whole body	+	+	Increased hepatic glycogen content, reduced adipose tissue mass	161
Gsk3b	Whole body (−/−)	NR	NR	Embryonic lethal	55
	Whole body (+/−)	NR	NR	Ameliorates genetically induced diabetes	55
	Pancreatic β-cells	NR	+	Increased pancreatic β-cell mass, protected against diet-induced diabetes	54
	Hepatocytes	UC	UC	No distinct metabolic phenotype	53
	Skeletal muscle	+	+	Increased muscle glycogen content	53
Tsc1	Pancreatic β-cells	−	+	Increased pancreatic β-cell mass, improved glycaemic control in young mice, obesity in old mice	56
	Hepatocytes	−	−	Protected against diet-induced hepatic steatosis	62
Tsc2	Pancreatic β-cells	NR	+	Increased pancreatic β-cell mass	57
Raptor	Skeletal muscle	NR	−	Increased muscle glycogen content, progressive muscle dystrophy	59
	Adipose tissue	NR	+	Protected against diet-induced obesity and hypercholesterolaemia	60
S6k	Whole body	+	−	Reduced pancreatic β-cell mass, hypoinsulinaemia, protected against age- and diet-induced obesity and insulin resistance	58, 61

Further descriptions are given in the text. NR, not reported; UC, unchanged; +, improved; −, reduced; (−/−), homozygous mutant; (+/−), heterozygous mutant.

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Table 2. Overview of mouse models for the role of Pten and Ptp1b in glucose homeostasis

Gene	Deleted in	Insulin sensitivity	Glucose tolerance	Further characteristics	Refs
Pten	Whole body (−/−)	NR	NR	Embryonic lethal	64
	Whole body (+/−)	+	+	Protected against genetically induced diabetes, spontaneous tumour development	64, 72, 162
	Pancreatic β-cells	NR	NR	Hypoglycaemia, hypoinsulinaemia, protected against streptozotocin- and diet-induced diabetes	71
	Skeletal muscle	UC	+	Protected against diet-induced insulin resistance and diabetes	67
	Adipose tissue	+	+	Resistant to streptozotocin-induced diabetes	68
	Hepatocytes	NR	+	Age-dependent hepatic steatosis and its progressive forms	69, 70
Ptp1b	Whole body	+	+	Protected against diet-induced diabetes	63
	Skeletal muscle	+	+	Protected against diet-induced insulin resistance	65
	Hepatocytes	+	+	Reduced hepatic lipid content after 5 weeks of a high-fat diet, protected against diet-induced insulin resistance	66

UC, unchanged; +, improved; −, reduced; (−/−), homozygous mutant; (+/−), hemizygous.

effects of mTOR inhibition in other diseases, such as pancreatic neuroendocrine tumours, astrocytomas, lymphangioleiomyomatosis and autosomal dominant polycystic kidney disease (Refs 79, 80, 81, 82, 83, 84). Owing to their inhibitory effect on proliferation of lymphocytes, both compounds have also been used for immunosuppression after transplantation.

However, several side effects have been reported, such as myelosuppression, pulmonary toxicity and metabolic disturbances (Refs 85, 86). Treatment of mRCC with mTOR inhibitors was associated with increased blood glucose levels, hypertriglyceridaemia and hypercholesterolaemia (Refs 76, 77). Similarly, the use of mTOR inhibitors after kidney transplantation was linked to elevated cholesterol and triglyceride levels compared with other immunosuppressive regimens and, thus, the subsequent need for lipid-lowering therapy (Ref. 87). Diabetes mellitus is a frequent complication after solid organ transplantation with an increased risk of graft failure and cardiovascular mortality. Whereas immunosuppressive treatments with glucocorticoids and calcineurin inhibitors are known to result in insulin resistance and impaired insulin secretion, respectively, the role of mTOR inhibitors in the development of diabetes after transplantation is more controversial (Refs 88, 89). Some studies indicate an independent association of mTOR inhibitors with diabetes onset after transplantation, but others did not come to the same conclusion (Refs 90, 91, 92).

Although mTOR inhibitors have been implemented successfully in different clinical settings, they may only be used in the treatment

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of specific tumour types, and their efficacy might be limited by cellular escape mechanisms such as rapamycin resistance (Ref. 77). Targeting multiple components of the PI3K/AKT pathway might improve antitumour potency and broaden the spectrum of susceptible tumour types. Indeed, based on structural similarities of PI3K and mTOR, newly developed inhibitors aimed at inhibition of both kinases simultaneously are currently under investigation. In addition to dual PI3K–mTOR inhibitors, selective AKT inhibitors are being tested in xenograft mouse models and early Phase I studies. However, inhibitors targeting multiple components might also have more severe side effects with regard to metabolic control. The use of techniques such as antibody-directed drug delivery could allow cell-type-specific targeting of the PI3K/AKT pathway and thus minimise side effects.

Stress response and MAPK signalling in acquired insulin resistance

Although at the core of the problem there is still no satisfying answer to the question of how insulin resistance develops, a widely discussed concept involves Ser/Thr kinases, which can phosphorylate numerous sites in IRS1 and IRS2. Phosphorylation of IRS proteins on Ser/Thr residues can uncouple the activated IR from downstream signal transduction modules (reviewed in Ref. 93). This phenomenon potentially depends on three different mechanisms: prevention of docking of IRS to IR, ubiquitylation followed by the proteolytic breakdown of IRS, or prevention of the docking of downstream effectors such as PI3K. Whereas in the former two cases all insulin-induced effects could be abolished, more selective defects might develop in the latter case, dependent on which modules are uncoupled from the activated IR. Multiple negative inputs converge at the level of IRS proteins. Of major importance appears to be the increased secretion of proinflammatory cytokines from adipocytes as observed in obesity. Proinflammatory signalling often involves activation of the inhibitor of κ light polypeptide gene enhancer in B-cells, kinase (IKBKB, IKK)–NF-κB axis, which is now regarded as a critical pathway linking obesity-associated chronic inflammation with insulin resistance. For example, tumour necrosis factor-dependent downregulation of IRS proteins depends on IKK and can be inhibited by aspirin (Ref. 94). Indeed, that salicylate can increase insulin sensitivity is an old observation (Ref. 95). Whereas *IKK*-knockout mice are embryonic lethal, mice with *IKK* hemizygosity show lower fasting blood glucose and insulin levels and improved free fatty acid levels relative to littermate controls when placed on a high-fat diet or rendered leptin deficient (Ref. 96). Furthermore, it has been shown that adipocyte-derived factors can act through IKK to induce insulin resistance in skeletal muscle (Ref. 97).

In addition to inflammation, the activation of Ser/Thr kinases with concomitant downregulation of the function of IRS proteins has been observed downstream of various conditions known to be associated with the development of insulin resistance and T2DM, such as hypoxia, endoplasmic reticulum (ER) stress and the generation of reactive oxygen species. Kinases activated under these conditions are also called stress kinases, because their activity positively correlates with the occurrence of imbalances in cellular homeostasis. An increase in circulating cytokines, as observed under systemic low-level inflammation during obesity, can also activate IRS Ser/Thr kinases (Ref. 93). Among the kinases targeting IRS are GSK3, S6K, p38 and several isoforms of the PKC family. The PKC family consists of 12 isoforms grouped as atypical PKCs (ζ and λ), conventional PKCs (α, β and γ), novel PKCs (δ, ϵ, η and θ), and protein kinase Ns (PKN1, PKN2 and PKN3), from which PKCδ, PKCλ/ζ and PKCθ are known to target IRS. One widely discussed case is the activation of JNK downstream of ER stress and the unfolded protein response (Refs 98, 99). Obese humans and rodents develop ER stress in hepatocytes and adipocytes, leading to JNK-dependent phosphorylation of IRS1 on Ser307 (numbering as in mouse) (Ref. 100) followed by its ubiquitylation and proteolytic breakdown. Indeed, global or conditional loss of JNK in adipose tissue, skeletal muscle or the brain was found to attenuate diet-induced insulin resistance in mice fed a high-fat diet, supporting the notion of a repressive role for JNK in insulin action (Ref. 9). Surprisingly, mice in which the target site for JNK in IRS1 (Ser307) was replaced by an alanine were less insulin sensitive, as were mice lacking JNK1 in hepatocytes (Refs 9, 101). The latter two observations indicate that JNK is required for insulin action in hepatocytes, once more underlining the context dependence of

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insulin signal transduction. The case of JNK exemplifies the dilemma: a significant number of IRS kinases believed to be responsible for the development of insulin resistance are also required for insulin-dependent metabolic control. For example, ERK1/2 are believed to link insulin with cell proliferation, differentiation and the regulation of lipid metabolism, whereas isoforms of PKC may be required for insulin-induced glucose transport (Refs 102, 103, 104, 105, 106, 107, 108, 109). These intricate interconnections certainly complicate the development of intervention strategies based on MAPKs in the treatment of insulin resistance.

AMPK – an energy sensor targeted in the treatment of metabolic syndrome

When intracellular energy levels are low, cellular metabolism must shift from energy-consuming anabolic processes towards energy-producing catabolic processes. AMPK, a sensor of the availability of intracellular energy, is activated at low energy levels and regulates cellular processes accordingly. This kinase inhibits insulin-stimulated anabolic processes such as de novo lipogenesis and glycogen synthesis. Nevertheless, AMPK activity supports whole-body glucose homeostasis and improves insulin sensitivity by promoting processes such as glucose uptake and energy expenditure. The effects of the widely used antidiabetic drug metformin have been shown to depend largely on activation of AMPK (Ref. 110). Thus, AMPK is currently the only protein kinase targeted in the treatment of metabolic syndrome.

AMPK signalling pathway

AMPK is a heterotrimeric complex consisting of a catalytic α-subunit and two regulatory subunits (β and γ). There are several isoforms of each subunit encoded by individual genes, including *PRKAA1* (α1), *PRKAA2* (α2), *PRKAB1* (β1), *PRKAB2* (β2), *PRKAG1* (γ1), *PRKAG2* (γ2) and *PRKAG3* (γ3) (Ref. 111). The different isoforms of AMPK subunits are expressed tissue specifically and exert both overlapping and distinct functions (Refs 112, 113). The AMPK pathway is activated by a variety of physiological stimuli, such as glucose deprivation, hypoxia, oxidative stress and muscle contraction. The common result of these stimuli is a reduction in cellular energy level and an increase in AMP/ATP ratio, which is crucial for AMPK activity. AMPK is also activated by different hormones, including leptin and adiponectin, but the mechanisms by which these hormones activate AMPK are not yet fully elucidated. For full kinase activity, AMPK must be phosphorylated at Thr172 in the catalytic domain of the α-subunit by upstream kinases such as serine/threonine kinase 11 (STK11, LKB1) and calcium/calmodulin-dependent protein kinase kinase β (CAMKKβ). LKB1 is a constitutively active kinase and considered to be the predominant upstream kinase of AMPK, but also phosphorylates 13 other AMPK-related kinases (Ref. 114). Protein phosphatases (PP2A and PP2C) antagonise upstream kinases and inhibit AMPK activity by dephosphorylation of Thr172. Most importantly, AMPK activity and Thr172 phosphorylation are highly dependent on the intracellular AMP/ATP ratio. AMP and ATP bind to the γ-subunit of AMPK in a competitive manner. When the AMP/ATP ratio is high, binding of AMP to AMPK allosterically activates kinase activity fivefold and induces conformational changes that block the dephosphorylation of Thr172 by PP2A and PP2C, which preserves activation by upstream kinases (reviewed in Refs 111, 115, 116). It has been recently proposed that binding of AMP triggers exposure of a myristoyl group at the AMPK β-subunit, which promotes membrane association and primes AMPK for activation by upstream kinases (Ref. 117). In addition, it was shown that binding of ADP to AMPK protects against dephosphorylation of Thr172, but does not induce allosteric activation of AMPK (Ref. 118). Activated AMPK phosphorylates substrates such as AS160, GYS1, acetyl-CoA carboxylase α (ACACA, ACC) and malonyl-CoA decarboxylase (MLYCD, MCD), thus stimulating glucose uptake, inhibiting glycogen synthesis, inhibiting de novo lipogenesis and enhancing β-oxidation, respectively. AMPK also indirectly inhibits mTORC1, thereby blocking protein synthesis, enhancing respiration and probably improving insulin sensitivity by counteracting mTORC1- and S6K-induced inhibition of IRS1/2 (reviewed in Ref. 116).

Complex role of AMPK isoforms in metabolic control

As mentioned above, the antidiabetic effects of metformin largely depend on AMPK activation. Thus, characterising the role of AMPK isoforms

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in mammalian physiology is of great importance and a prerequisite for the achievement of more specific and efficient targeting of AMPK compared with metformin. Genetic mutations in elements of the AMPK pathway in humans and their pathophysiological effects in glucose homeostasis are not yet fully characterised. Several polymorphisms in LKB1 and AMPK α2 and γ2 subunits are associated with insulin resistance and T2DM in different subsets of patients (Refs 119, 120, 121). Interestingly, polymorphisms in LKB1, α1-, α2- and β2-subunits of AMPK as well as in AMPK targets myocyte enhancer factor 2A (MEF2A) and MEF2D were found to be associated with reduced response to metformin treatment (Refs 119, 121). Because metformin is thought to function mainly by activating AMPK, the identified polymorphisms might affect the functions of LKB1, AMPK, MEF2A and MEF2D. However, the physiological and biochemical consequences of the identified polymorphisms remain to be characterised. Apart from that, LKB1 has tumour suppressor functions and its mutation can cause Peutz–Jeghers syndrome, which is characterised by mucocutaneous pigmentation, hamartomatous polyps and increased risk of cancer (Ref. 122). In addition, mutations in *PRKAG2* were shown to cause hypertrophic cardiomyopathy with Wolff–Parkinson–White syndrome owing to a glycogen storage disorder (Refs 123, 124, 125).

The complex roles of AMPK isoforms in insulin-sensitive tissues have been studied in transgenic mice. Whereas loss of AMPKα1 did not alter metabolic control in mice, global or tissue-specific loss of individual AMPK isoforms mostly led to impaired glucose homeostasis (Ref. 126). PRKAα2-knockout mice were glucose intolerant and insulin resistant and showed impaired glucose uptake on stimulation with the AMPK activator 5-aminoimidazole-4-carboxamide riboside (AICAR) (Refs 10, 126). In addition, deletion of *Prkaa2* specifically in β-cells resulted in defective glucose-stimulated insulin secretion (Ref. 127). Hepatocyte-specific deletion of *Prkaa2* in the liver revealed that AMPK inhibits gluconeogenesis and release of glucose in the liver (Ref. 128). PRKAβ2-knockout mice had reduced maximal and endurance exercise capacities and were more susceptible to diet-induced weight gain and glucose intolerance; PRKAγ3-knockout mice were shown to have impaired AICAR-stimulated glucose uptake (Refs 129, 130). By contrast, activation of AMPK in the hypothalamus increased food intake, suggesting that inhibition of AMPK in the hypothalamus could protect against obesity-induced insulin resistance (Ref. 131). Indeed, mice lacking AMPKβ1, which is highly expressed in the liver and brain, were protected against diet-induced obesity, insulin resistance and hepatic steatosis, probably because of reduced food intake (Ref. 132).

These studies show that the effects of AMPK on glucose homeostasis are highly complex as a result of isoform- and tissue-specific functions. Simultaneous modulation of its activity in different tissues can have opposing effects on glucose homeostasis, which could complicate the development of therapeutic approaches directly targeting AMPK. However, isoform- and tissue-specific targeting could also provide a basis for highly specific and effective therapeutic approaches in addition to metformin treatment.

Metformin and AMPK in clinical use

Metformin has been used in the clinic for several decades for the treatment of insulin-resistant and diabetic patients. The drug improves insulin sensitivity, lowers blood glucose and cholesterol levels without risk of acute hypoglycaemia and weight gain, and reduces the risk of diabetes-related complications such as cardiovascular disease (Ref. 133). The notion that metformin elicits its beneficial effects mainly through the activation of AMPK is further underlined by observations of mice with abolished hepatic AMPK activity due to hepatocyte-specific LKB1 deficiency (Ref. 134). Metformin failed to lower blood glucose in these mice, indicating that activation of AMPK through LKB1 in the liver is required (Ref. 134). Nevertheless, several AMPK-independent effects of metformin have been reported (Ref. 110). It was recently shown that metformin can block gluconeogenesis in isolated mouse hepatocytes independently of LKB1 and AMPK (Ref. 135).The mechanism of AMPK activation by metformin is still controversial. One hypothesis is that metformin activates AMPK indirectly by inhibiting complex I of the respiratory chain, which compromises cellular energy production and increases the AMP/ATP ratio (Refs 136, 137). However, metformin also activates AMPK in an

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adenine-nucleotide-independent manner (Ref. 138). More recently, it was proposed that metformin activates PKCζ, which phosphorylates LKB1 at Ser428, resulting in nuclear export of LKB1 and activation of AMPK (Ref. 139). Metformin may mainly activate AMPK in the liver, muscle and vasculature, because cellular uptake of the drug is dependent on transmembrane transporters such as solute carrier family 22 (organic cation transporter), member 1 (SLC22A1, OCT-1). Whereas OCT-1-deficient mice indeed have a diminished response to metformin, the role of OCT-1 polymorphisms in diabetic patients is controversial (Refs 140, 141).

Metformin is used at inconveniently high doses, and its clinical use is restricted in patients with renal or hepatic disease owing to increased risk of lactic acidosis (Ref. 133). Hence, direct activation of AMPK by other means would be an attractive alternative in the treatment of diabetic patients. The AMPK activator A-769662 efficiently lowered blood glucose and triglycerides and transiently reduced body weight gain in mouse models of genetically induced obesity and insulin resistance (Ref. 142). In addition, treatment with AICAR was shown to reduce blood glucose levels in diabetic patients. One side effect associated with the activation of AMPK could be increased food intake because of its role in the hypothalamus. However, treatment with metformin reduces body weight in patients by decreasing appetite and food intake (Refs 143, 144). The underlying mechanisms remain poorly understood (Refs 143, 144). A transient reduction in food intake was reported in obese mice, but not in lean mice treated with A-769662, because this drug may not activate AMPK in the brain (Ref. 142). By contrast, increased food intake was observed in mice treated with AICAR (Refs 131, 145). A-769662 and AICAR were also shown to have AMPK-independent activity, and possible side effects of long-term treatment have not been assessed (Refs 146, 147).

Metformin is now also considered for use in cancer therapy. Epidemiological studies have assessed the association between obesity or T2DM and cancer in large populations (Refs 148, 149). Although intensively investigated, the molecular mechanisms linking cancer with obesity are still not fully elucidated. Chronic hyperinsulinaemia has been suggested to contribute to increased tumour growth, because it may directly activate insulin receptor on (pre-)neoplastic cells or indirectly through promotion of insulin-like growth factor 1 (IGF1) synthesis. Both insulin and IGF1 enhance tumour growth in xenograft models by increasing cell proliferation and inhibiting apoptosis. There is an ongoing debate as to whether the use of insulin analogues in the treatment of obese and diabetic patients could further increase the risk of cancer. Whereas certain insulin analogues do lead to tumour development in rats, their effect in human patients remains controversial (Refs 150, 151, 152). In line with the amelioration of obesity and hyperinsulinemia by metformin, observational data showed that its use was associated with a reduced risk of cancer (Refs 143, 153, 154). Additionally, it might also inhibit tumor progression by AMPK-mediated inhibition of mTORC1, and possibly also by a Rac GTPase-dependent and AMPK-independent mechanism (Ref. 155). Combined cancer therapy with metformin and drugs targeting the PI3K/AKT pathway might result in the synergistic inhibition of mTORC1. This strategy could also overcome impaired glucose homeostasis resulting from PI3K/AKT pathway inhibition.

Concluding remarks

The insulin signal transduction network and the biochemical properties of its components have been extensively studied. There is increasing knowledge of how PI3K/AKT, MAPK and AMPK signalling controls and how their failure impairs glucose homeostasis. Moreover, studies in transgenic mice have demonstrated that specific modulation of protein kinase signalling can effectively improve glucose homeostasis and protect against obesity, acquired insulin resistance and diabetes. However, very little translation into clinical practice has taken place. Metabolically relevant cellular functions such as glucose transport, lipogenesis, glycogen synthesis and gluconeogenesis are controlled by kinases that do not act exclusively within the insulin signal transduction network. It has emerged that all signal transduction events within a cell are interconnected and that mere description of the network is not sufficient to define mechanisms underlying both context and stimuli specificity.

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Hence, global modulation of kinase activity by, for example deletion of PTEN and the use of mTOR inhibitors might result in severe side effects such as cancer and impaired metabolic control, respectively. The development of safe kinase-based therapies will probably remain elusive until we understand how cells integrate signalling information to implement context in their respective intracellular signal transduction network. In addition, the development of specific inhibitors is complicated by high structural similarities in the catalytic domains of different protein kinases. Reduced specificity resulting in the inhibition of multiple targets could be beneficial in cancer therapy because it might potentiate toxicity on cancer cells. Inhibitors used for the treatment of metabolic syndrome should, by contrast, be highly specific in order to minimise side effects and allow long-term treatment. Targeting kinases in their inactive state, in which they show higher structural diversity than in their active conformation, or disrupting protein complexes of kinases was suggested for the design of inhibitors with increased specificity (Refs 156, 157, 158). Tissue-specific targeting by using transmembrane carriers or metabolic activation, as well as the targeting of specific isoforms or effectors further downstream, might provide a route to increased specificity of drugs and minimal side effects.

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Features associated with this article

Figure

Figure 1. Simplified view of insulin-stimulated PI3K/AKT signalling and its substrates involved in cellular metabolism.

Tables

Table 1. Overview of mouse models for AKT isoforms and downstream targets.

Table 2. Overview of mouse models for the role of Pten and Ptp1b in glucose homeostasis.

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6.2. Promiscuous affairs of PKB/AKT isoforms in metabolism.

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Abstract

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RESEARCH ARTICLE

Promiscuous affairs of PKB/AKT isoforms in metabolism

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Abstract

The protein kinase B (PKB) family encompasses three isoforms; PKBα (AKT1), PKBβ (AKT2) and PKBγ (AKT3). PKBα and PKBβ but not PKBγ, are prominently expressed in classical insulin-sensitive tissues like liver, muscle and fat. Transgenic mice deficient for PKBα, PKBβ or PKBγ have been analysed to study the roles of PKB isoforms in metabolic regulation. Until recently, only loss of PKBβ was reported to result in metabolic disorders, especially insulin resistance, in humans and mice. However, a new study has shown that PKBα-deficient mice can show enhanced glucose tolerance accompanied by improved β-cell function and higher insulin sensitivity in adipocytes. These findings prompted us to review the relevant literature on the regulation of glucose metabolism by PKB isoforms in liver, skeletal muscle, adipocytes and pancreas.

Keywords: *PKB; AKT; redundancy; metabolism; glucose; liver; muscle; fat; pancreas*

Introduction

The level of circulating glucose has to be adjusted to variations in food intake and energy demands, which is primarily regulated by the insulin/glucagon system in mammals. Insulin lowers blood glucose by increasing uptake and deposition into muscle and adipose tissue as well as by decreasing its release from the liver. Glucagon mainly acts on hepatocytes where it opposes the action of insulin and stimulates release of glucose into circulation. Insulin signalling targets various cell types in the whole organism and, in addition to its role in metabolism, affects cellular processes such as protein synthesis, proliferation and survival. Therefore a complex network of molecular pathways is required to transduce insulin signalling into cell type-specific and context-dependent responses. Protein kinase B (PKB) has been shown to be a key element in the insulin signal transduction network.

The physiological and tissue-specific effects of the three PKB isoforms have been extensively studied *in vitro* but most comprehensively in transgenic mice. While *Pkbα*$^{-/-}$ and *Pkbγ*$^{-/-}$ mice show impaired foetal growth and brain development, respectively, glucose homeostasis was found unaffected in both models (Chen *et al.*, 2001; Cho *et al.*, 2001b; Easton *et al.*, 2005; Tschopp *et al.*, 2005; Yang *et al.*, 2003). In contrast, *Pkbβ*$^{-/-}$ mice are insulin resistant, mildly glucose-intolerant and have less adipose tissue. Depending on strain and gender, these mice show either late loss of β-cells followed by development of diabetes and mild growth deficiency, or compensatory increase of β-cell mass without age-dependent progression into overt hyperglycaemia (Cho *et al.*, 2001a; Garofalo *et al.*, 2003). These studies suggested that only PKBβ plays a role in regulation of energy homeostasis. This view was recently challenged by a new study (Buzzi *et al.*, 2010) that re-examined in parallel the metabolic phenotypes of three mouse strains deficient for Pkbα, β or γ, respectively. Here they confirmed that *Pkbβ*$^{-/-}$ mice are insulin resistant with compensatory increase of islet mass and that *Pkbγ*$^{-/-}$ mice show no metabolic

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abnormalities. However, *Pkbα*$^{-/-}$ mice displayed improved insulin sensitivity, lower blood glucose and higher serum glucagon concentrations. These new findings prompted us to critically review the relevant literature on the metabolic role of PKB isoforms.

Protein Kinase B

The PKB serine/threonine protein kinase family consists of three evolutionary conserved isoforms: PKBα (AKT1), PKBβ (AKT2) and PKBγ (AKT3). PKB was first isolated from transforming murine leukaemia virus AKT-8 by Staal *et al* in 1977, characterized as an oncogene and named *akt* (Staal *et al.*, 1977). Two human homologues of the viral *akt* gene were identified later on and termed *akt1* and *akt2*. In 1991, a human serine/threonine kinase was cloned, which was termed *related to the A and C kinases* (RAC) and subsequently renamed to PKBα/Akt1 (Jones *et al.*, 1991, reviewed in Brazil and Hemmings, 2001). Since the identification of PKBα as a serine/threonine kinase, almost 20 years ago, PKB isoforms were studied intensively and are now considered as major regulators of elementary cellular process, such as proliferation, survival, cell growth and energy metabolism (Bozulic *et al.*, 2008; Contreras-Ferrat *et al.*, 2010; Haga *et al.*, 2005; Heron-Milhavet *et al.*, 2006). Consequently, PKB isoforms play pivotal roles in physiology and their deregulation engenders diseases, such as cancer, neurodegeneration and metabolic disorders (Altomare and Testa, 2005; Zhao and Townsend, 2009). Remarkably, activation of PKB isoforms by gene amplification or mutations in upstream regulators frequently occurs in human cancers (Carpten *et al.*, 2007). The molecular and cellular biology of PKB isoforms has been comprehensively reviewed (Brazil *et al.*, 2002; Hanada *et al.*, 2004; Manning and Cantley, 2007) and is therefore discussed only briefly in this review.

PKBα, PKBβ and PKBγ are encoded by three distinct genes, which are located on different chromosomes. In contrast to many members of other kinase families, PKB isoforms share the same protein structure and are approximately 80% identical at the amino acid level. They carry a N-terminal pleckstrin homology (PH) domain, a catalytic domain and a C-terminal regulatory domain (Hanada *et al.*, 2004). PKBα and PKBβ are ubiquitously expressed, whereas PKBγ expression is restricted to brain, testis, lung, fat, mammary glands and pancreatic islets (Buzzi *et al.*, 2010; Yang *et al.*, 2003). Low expression levels were also observed in skeletal muscle (Brozinick *et al.*, 2003). Notably, expression of PKBα and PKBβ is prominent in classical insulin target tissues involved in the regulation of systemic energy homeostasis, such as liver, skeletal muscle and fat (Yang *et al.*, 2003).

PKB isoforms are activated by growth factors and cytokines, including PDGF, VEGF, HGF, IGF-1, insulin, TNFα and IL-2 but also by environmental stresses, such as heat shock, hypoxia and oxidative stress (Zhuravleva *et al.*, 2010). Regulation of PKB downstream of these stimuli generally depends on activation of phosphatidyl-inositol 3-kinase (PI3K) family members, which convert phosphatidy-inositol di-phosphate (PIP2) to PIP3 at the plasma membrane. PKB isoforms bind to PIP3 via their PH domain, which facilitates their activation. PKB isoforms are phosphorylated and thereby activated by upstream kinases at two distinct phosphorylation-sites. First, PDK1 phosphorylates PKB isoforms at the catalytic domain (Thr308 in PKBα, Thr309 in PKBβ and Thr305 in PKBγ) which results in basal kinase activity of approximately 10%. In a second step, PKB isoforms are phosphorylated by mTORC2, DNA-PK or ATM at the C-terminal regulatory domain (Ser473 in PKBα, Ser474 in PKBβ and Ser472 in PKBγ), which is essential for full kinase activity. Activation of PKB isoforms is tightly counter-regulated by phosphatases, such as PTEN and SHIP2. These phosphatases inactivate PIP3 by dephosphorylation and thereby prevent plasma membrane translocation and activation of PKB isoforms (Bhaskar and Hay, 2007; Brazil and Hemmings, 2001; Hanada *et al.*, 2004; Zhuravleva *et al.*, 2010).

Activity of PKB isoforms is modulated by different binding partners, such as TRAF6, HSP90, CTMP and TCL1 which affect protein stability, dynamics and duration of activation and rate of kinase activity, respectively (Brazil *et al.*, 2002). Furthermore, recent studies show that activity of PKB isoforms is indirectly modulated by several microRNAs. For instance, miR320 was shown to down-regulate PI3K in adipocytes resulting in inhibition of PKB activation and insulin resistance (Ling *et al.*, 2009). However, it remains to be determined if modulation of PKB activity by binding partners and microRNAs plays a role in insulin signalling and regulation of glucose homeostasis.

Upon activation, PKB isoforms are released from the plasma membrane and phosphorylate various substrates throughout the cell. PKB substrates can specifically regulate a single respective cellular process (e.g. cell survival, Bad or caspase 9), or pleiotropically affect several cellular functions simultaneously, such as GSK3β and FoxO transcription factors which can control cell survival, proliferation but also energy metabolism (Manning and Cantley, 2007)

The different phenotypes of *Pkbα*$^{-/-}$, *Pkbβ*$^{-/-}$ and *Pkbγ*$^{-/-}$ mice undoubtedly point to isoform-specific

functions. As the expression of PKB isoforms overlaps in many organs, the different phenotypes can not solely be explained by divergent gene expression. There is an emerging number of studies describing isoform-specific functions in cellular processes, such as PKBα in cancer cell migration (Chin and Toker, 2010), PKBα in β-cell proliferation and PKBβ in glucose uptake. Since PKB isoforms are structurally highly similar, including the kinase domain, it is unlikely that recognition of phosphorylation-motifs underlies substrate-specificity (Manning and Cantley, 2007). Therefore it is considered that substrate-specificity is controlled by cellular localization and specific binding partners. However, until today, functional differences of PKB isoforms at the cellular level are not yet fully characterized and the mechanisms determining substrate-specificity remain largely unknown.

Liver

The liver functions as a critical regulator of glucose homeostasis and the PI3K/PKB pathway co-ordinates hepatic glucose metabolism with the systemic metabolic state. Out of the three PKB isoforms, only PKBα and PKBβ, but not PKBγ, are expressed in the liver. Studies using transgenic mice have shown, that PKBβ accounts for approximately 70% of total PKB protein in the liver and is therefore considered as the major isoform (Dummler *et al.*, 2006). In hepatocytes, PKBα and PKBβ are both activated upon insulin stimulation in a PI3K-dependent manner (Taniguchi *et al.*, 2006).

According to the current model, insulin suppresses hepatic glucose output in several ways, including inhibition of gluconeogensis and stimulation of glycogen synthesis, which are both dependent on PKB activity (Newgard, 2003). Gluconeogenesis is suppressed after phosphorylation/inhibition of the transcription factor FoxO1, an inducer of gluconeogenic genes *pepck* and *g6pase* (Taniguchi *et al.*, 2006). In addition, PKB also induces glycogen synthesis by phosphorylating and inhibiting GSK3β (Lawrence and Roach, 1997). Although it was shown that PKBβ (He *et al.*, 2010, Leavens *et al.*, 2009) plays a critical role in these processes, the effects of endogenous PKBα remain unclear. Furthermore, it was proposed that hepatic *de novo* lipogenesis is mainly regulated by PKCλ/ζ, as expression of lipogenic genes, such as *srebp-1c*, is depended on PKCλ/ζ activity (Taniguchi *et al.*, 2006). Even so, several studies show that PKBα and PKBβ promote hepatic *de novo* lipogenesis as well (He *et al.*, 2010; Leavens *et al.*, 2009; Ono *et al.*, 2003).

The role of PKB activity in hepatic metabolism and its effects on systemic energy homeostasis was studied in mice with liver-specific deletions of major regulatory subunits of PI3K ($pik3r1^{\Delta li}/pik3r2^{\Delta li}$) and PTEN ($pten^{\Delta li}$) (Horie *et al.*, 2004; Stiles *et al.*, 2004; Taniguchi *et al.*, 2006). Insulin-stimulated activation of PKBα, PKBβ and PKCλ/ζ was almost completely abrogated in $pik3r1^{\Delta li}/pik3r2^{\Delta li}$ mice. Concomitantly, insulin also failed to down-regulate hepatic gluconeogenesis and could no longer inhibit GSK3β and induce expression of lipogenic genes. As a consequence, $pik3r1^{\Delta li}/pik3r2^{\Delta li}$ mice exhibited insulin resistance, hyperglycaemia, hyperinsulinaemia and were glucose intolerant (Taniguchi *et al.*, 2006). Remarkably, expression of gluconeogenic genes, *pepck* and *g6pase* was efficiently blocked after over-expression of constitutive active PKBα (myr-PKBα) whereas expression of lipogenic genes, *srebp-1c*, could only be restored by over-expression of constitutive active PKCλ/ζ (Taniguchi *et al.*, 2006). On the other hand, gluconeogenic genes were down regulated and phosphorylation of GSK3β and lipogenesis were enhanced in liver of $pten^{\Delta li}$ mice, most likely due to hyper-activated PKBα, PKBβ and PKCλ/ζ. As a result, $pten^{\Delta li}$ mice were found to be hypoglycaemic, hypoinsulinaemic, showed increased glucose tolerance and, most strikingly, developed hepatic steatosis with all characteristics of human non-alcoholic fatty liver disease (Horie *et al.*, 2004; Stiles *et al.*, 2004).

In recent studies the role PKBβ in hepatic lipid accumulation was examined using different mouse models of hepatic steatosis (He *et al.*, 2010; Leavens *et al.*, 2009). Remarkably, whole-body deletion of *pkbβ* in $pten^{\Delta li}$, leptin-deficient ($lep^{ob/ob}$) and mice on high-fat diet (HFD) as well as liver-specific deletion of *pkbβ* in $lep^{ob/ob}$ and mice on HFD significantly reduced lipid accumulation in hepatocytes. PKBβ-deficiency reduced expression of lipogenic genes and *de novo* lipogenesis, indicating that PKBβ is required for lipid accumulation in hepatocytes.

Interestingly, *pkbβ*-deficiency had more pronounced effects in wildtype controls compared to $pten^{\Delta li}$ mice. Therefore, increased activation of PKBα, and possibly also of PKCλ/ζ, might compensate for loss of PKBβ in hepatocytes. Indeed ectopic expression of constitutively active PKBα (myr-PKBα) in the liver induced hypoglycaemia and hepatic steotosis, further supporting the notion for functional overlap between PKBα and PKBβ in hepatocytes (Ono *et al.*, 2003). However, activation of PKB isoforms by myristilation is rather artificial, and might induce non-physiological functions.

Notably, hepatic lipid content, but not *de novo* lipogenesis or expression of lipogenic genes, were reduced in PKBβ –deficient mice fed a specific HFD (*Surwit diet*; Leavens *et al.*, 2009). Moreover, expression of

myr-PKBα upregulated *srebp-1c* in wildtype but not *pik3r1*$^{\Delta li}$/*pik3r2*$^{\Delta li}$ mice and additionally promoted accumulation of hepatic lipids independent of srebp-1c (Ono *et al.*, 2003; Taniguchi *et al.*, 2006). These observations suggest that PKBα and PKBβ regulate other processes in addition and that reduced lipogenesis could be a secondary effect dependent on PI3K/PKC.

Skeletal muscle

Skeletal muscle is a specialized tissue that makes movement possible by transforming chemical energy into mechanical force. In addition, skeletal muscle is also central to metabolic regulation and 70 to 90% of glucose disposal during a hyperinsulinaemic euglycaemic clamp occurs in this tissue (DeFronzo *et al.*, 1981). Glucose taken up is mainly incorporated into glycogen (Shulman *et al.*, 1990) and the majority (≈80%) of carbohydrates stored in humans are found in skeletal muscle (Jensen and Lai, 2009).

It is the generally accepted view that insulin activates PKB via class 1A PI3K (Shepherd, 2005). Although this has not been conclusively shown in fully differentiated skeletal muscle, all available data support the view that PKB promotes insulin-stimulated glucose uptake and glycogen synthase activation (Cleasby *et al.*, 2007). As in adipose tissue, glucose uptake is increased in skeletal muscle by triggering translocation of GLUT4 from intracellular vesicles to the plasma membrane, which depends on well studied signalling events downstream of the insulin receptor involving IRS, PKB and AS160. Skeletal muscle expresses all three PKB isoforms (Brozinick *et al.*, 2003; Turinsky and Damrau-Abney, 1999) but only deletion of PKBβ causes insulin resistance and reduces insulin-stimulated glucose uptake (Cho *et al.*, 2001a; Garofalo *et al.*, 2003), indicating that PKBβ is required for this process. However, since high concentrations of insulin could still increase glucose disposal into muscle lacking PKBβ other signalling components might also be able to regulate GLUT4 translocation downstream of insulin. Indeed, over-expression of constitutively active PKBβ increases glucose uptake in L6 muscle cells (Hajduch *et al.*, 1998) suggesting that PKBα and PKBβ can both regulate translocation of GLUT4 to the plasma membrane. This finding is in line with the observation that insulin can activate all three isoforms of PKB in skeletal muscle (Brennesvik *et al.*, 2005; Brozinick *et al.*, 2003). Isoform-specific function has been investigated in several studies. Ectopic expression of constitutively active PKBα or PKBβ *in vivo* in rat muscle fibres increased glycogen accumulation, but only expression of PKBβ increased basal glucose uptake (Cleasby *et al.*, 2007). However, only PKBα increased glycogen synthase kinase-3beta (GSK3β) phosphorylation. Knockdown of PKBβ in fully differentiated muscle fibres by electrotransfer of short hairpin (sh)-RNAs decreased insulin-stimulated glucose uptake suggesting isoform-specificity of PKB in regulation of glucose metabolism (Cleasby *et al.*, 2007). Unfortunately, the role PKBα was not addressed in this study. Interestingly, Brozinick *et al.* (2003) found that insulin-stimulated PKBβ activation was reduced in insulin resistant muscles whereas insulin-stimulated activation of PKBα and PKBγ occurred normally. Evidence for isoform-specific signalling was provided by Bouzakri *et al.* (2006). These authors could show that activation of PKBβ via IRS1 stimulates glucose uptake whereas IRS2-mediated activation of PKBα increases lipid synthesis.

Besides insulin, muscle contraction can also induce glucose uptake. Contraction-induced glucose transport depends on increased translocation of GLUT4 but the underlying mechanism depends on AMPK and not on PI3K. However, contraction can increase PKB phosphorylation and activity (Sakamoto *et al.*, 2003; Whitehead *et al.*, 2000), but contraction-mediated PKB phosphorylation remains below 10% of insulin-stimulated PKB phosphorylation (Whitehead *et al.*, 2000). Contraction increases activity of all isoforms, but PKBα activation is most pronounced (Sakamoto *et al.*, 2002). The notion that PKB does not mediate contraction-stimulated glucose uptake is in line with the fact that the PI3K inhibitor wortmannin does not inhibit contraction-stimulated glucose uptake (Whitehead *et al.*, 2000). Accordingly, contraction-stimulated glucose transport is normal in skeletal muscle of PKBβ-deficient mice (Sakamoto *et al.*, 2006). Interestingly, contraction can block insulin-stimulated class 1A PI3K activity without reducing insulin-stimulated PKB activation (Whitehead *et al.*, 2000) indicating, that PKB phosphorylation might occur without increase in class 1A PI3K activity. This observation highlights the need to clarify which isoform(s) of PI3K mediate insulin-stimulated glucose uptake in skeletal muscle.

Adipose tissue

Insulin induces phosphorylation of PKB in adipocytes. Like in muscle and liver, the level of phosphorylation/activation of PKB after insulin stimulation is often regarded as benchmark for insulin sensitivity. This consensus is based on observations, that insulin resistance in adipose tissue is in many cases associated with less insulin-induced phosphorylation of PKB and that increased or constitutive activation of PKB

can increase or mimic insulin action, respectively. Many lines of evidence suggest, that mainly PKBβ is required downstream of insulin in adipocytes. For example, transient down-regulation of PKBβ using siRNAs inhibits insulin-induced GLUT4 translocation to the plasma membrane of 3T3-L1 adipocytes whereas knockdown of PKBα did not result in any differences (Jiang *et al.*, 2003; Katome *et al.*, 2003). These findings are in line with the observation that *Pkbβ*$^{-/-}$ mice are insulin resistant whereas *Pkbα*$^{-/-}$ mice are normal or even more insulin sensitive (Buzzi *et al.*, 2010; Chen *et al.*, 2001; Cho *et al.*, 2001a, b; Garofalo *et al.*, 2003; Yang *et al.*, 2003). However, not all metabolic functions regulated by insulin in adipocytes might be dependent on PKBβ alone, as described by Katome *et al.* (2003). These authors analysed 2-DG uptake and glycogen synthesis in 3T3-L1 adipocytes after down-regulation of PKBα, β or γ and found that PKBα and PKBβ contributed to insulin-stimulated glycogen synthesis to about the same extent while 2-DG uptake only depended on PKBβ. Accordingly, insulin appears to stimulate the association of PKBβ with GLUT4-containing vesicles in rat adipocytes (Calera *et al.*, 1998) and over-expression of PKBβ, but not PKBα, rescues impaired glucose transport in PKBβ-deficient adipocytes (Bae *et al.*, 2003). How isoform-specificity might be achieved in adipocytes was studied by Gonzales and McGraw (2009). These authors found that insulin activates both PKBα and PKBβ in adipocytes, but describe differential subcellular distribution of these two isoforms upon stimulation. While basally adipocytes had similar levels of PKBα and PKBβ at the plasma membrane, a significantly greater fraction of PKBβ accumulated at the plasma membrane after stimulation with insulin. The question if PKB plays a role in lipogenesis has received surprisingly little attention. However, Berggreen *et al.* (2009) recently described that inhibition of PKB in 3T3-L1 adipocytes with an inhibitor called Akti reduced *de novo* and insulin-dependent lipid synthesis and that insulin failed to regulate the rate-limiting lipogenic enzyme acetyl-CoA carboxylase (ACC) when PKB was inhibited. Specific roles for the different isoforms of PKB were not described in this study.

Interestingly, there is a small but noteworthy number of studies with conflicting results. For example, Kitamura *et al.* (1998) expressed a dominant-negative PKB isoform (Akt-AA) in 3T3-L1 adipocytes. This isoform contains two alanines instead of the two regulated phosphorylation sites (Thr308 and Ser473) and its expression reduced activation of PKB by about 80–95%. Expression of Akt-AA inhibited insulin-dependent protein synthesis without affecting glucose transport indicating, that PKB might only be required for some but not all effects of insulin in adipocytes. Similarly, Guilherme and Czech (1998) presented evidence that the formation of IRS1/PI3K complexes and Akt/PKB activation are insufficient to stimulate glucose transport in rat adipocytes. At least two more recent studies also describe that insulin-dependent activation of PKB does not necessarily correlate with insulin-induced 2-DG transport (Hoehn *et al.*, 2008; Xu *et al.*, 2010). Finally, Buzzi *et al.* (2010) found that primary adipocytes isolated from *Pkbα*-deficient mice show higher insulin-induced glucose incorporation than adipocytes from wild type littermates.

Pancreatic islets

To proper regulate blood glucose homeostasis islet mass and function has to be co-ordinated with metabolic demand. Plasticity of islet mass is achieved by integration of a complex signal environment comprised of nutrients, hormones and cytokines that controls the balance between apoptosis and cell growth/proliferation (Maedler, 2008; Niessen, 2006). Because PKB is a global regulator of growth, proliferation and apoptosis, it has been implicated to play a major role in modulating plasticity of islet mass downstream of insulin receptor substrate 2 (IRS2) (Elghazi *et al.*, 2007; Hennige *et al.*, 2003; Kubota *et al.*, 2000; Lingohr *et al.*, 2003; Mohanty *et al.*, 2005; Park *et al.*, 2006; Takamoto *et al.*, 2008; Withers *et al.*, 1998; Wrede *et al.*, 2002). Yet, a number of studies addressing this issue yielded somewhat unexpected results. *Pkbα*-deficient mice show impaired placental development and foetal growth (Buzzi *et al.*, 2010; Chen *et al.*, 2001; Tuttle *et al.*, 2001; Yang *et al.*, 2003) but normal islet growth and function. *pkbβ*-deficient mice show impaired overall growth but display, dependent on strain and sex, even compensatory increase in β-cell mass (Buzzi *et al.*, 2010; Cho *et al.*, 2001a; Garofalo *et al.*, 2003). Finally, *pkbγ*-deficient mice display reduction in brain size without any distortions of islet function or mass (Buzzi *et al.*, 2010; Easton *et al.*, 2005; Tschopp *et al.*, 2005). In another study β-cell-specific loss of function for PKB was induced by expression of a kinase-dead dominant-negative form of PKBα (rip-*kdpkb*), however, only defective insulin secretion but no reduction in islet size was observed (Bernal-Mizrachi *et al.*, 2004). Since the dominant-negative form antagonizes all three isoforms this latter finding makes compensation between isoforms an unlikely explanation for the normal islet phenotypes of PKB-deficient mice. In contrast to the loss of function phenotype, ectopic expression of constitutively active PKBα under the control of the rat insulin promoter (rip) (Bernal-Mizrachi *et al.*, 2001; Tuttle *et al.*, 2001)

resulted in hypertrophy and hyperplasia of islets. Such mice were hyperinsulinaemic and resistant to streptozotocin-induced diabetes. Taken together the results from these mouse models suggest that although none of the PKB isoforms is required for maintenance of islet mass, constitutive activation of at least PKBα is sufficient to increase islet size. In order to reconcile these observations it was proposed (Niessen, 2006) that maintenance and compensatory expansion of islet mass (as observed in insulin resistance) do not depend on the same signal transduction pathways downstream of IRS2. This model predicts that PKB is only required for expansion but not for maintenance of islet mass. Which of the three PKB isoforms is/are required to regulate islets mass was studied recently by Buzzi *et al*. (2010). This study shows that only PKBα, but not PKBβ or γ, is specifically activated downstream of IRS2 in β-cells. Furthermore, adenoviral over-expression of PKBα increased proliferation of β-cells while over-expression of the remaining two isoforms was ineffective, indicating that PKBα is in control of the regulation of β-cell mass.

Perspectives and conclusions

Insulin sensitivity manifests at the signalling level and at the level of cellular function. Since the insulin receptor is present on many, if not all, mammalian cells the biological function of insulin is cell type-specific, and, within a given cell type, insulin often controls more than one cellular process. For example, insulin induces GLUT4-dependent transport and deposition of glucose into fat. It also inhibits lipolysis. The analysis of any of these endpoints after stimulation with insulin allows unambiguous determination of how insulin sensitive the target cell is for the respective function. It has become common practice in the field to correlate insulin-induced cellular effects with intracellular insulin signal transduction, however, insulin sensitivity at the signalling level is not easy to measure because the insulin receptor connects to an intricate and highly context-specific intra-cellular network of signalling molecules. In practice insulin-dependent activation of few protein kinases within this network is usually correlated with specific insulin-induced cellular responses. PKB is regarded as most important mediator of metabolic insulin action and its activation is often monitored by using phospho-specific antibodies in combination with Western blotting. However, as described in this review, insulin signalling might branch at the level of PKB isoforms to control different aspects of metabolic regulation but in most of the cases, the possibility for non-redundant roles of PKB isoforms is not taken into consideration. Technically, due to high conservation of the amino-acid sequence surrounding the phosphorylated Ser and Thr residues in the three isoforms, none of the available phospho-specific antibodies can be used to determine which isoform(s) is (are) activated without prior isoform-specific immunoprecipitation. As more and more evidence for specific and possibly even opposing roles of PKB isoforms accumulates it appears justified to reconsider the appropriateness of detecting PKB phosphorylation to assess overall insulin sensitivity without consideration of the specific isoform.

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Declaration of interest

The authors report no conflicts of interest.

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6.3. Liver Failure After Extended Hepatectomy in Mice Is Mediated by a p21-Dependent Barrier to Liver Regeneration

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Abstract

Background & Aims: Extended liver resection leads to hepatic failure because of a small remnant liver volume. Excessive parenchymal damage has been proposed as the principal cause of this failure, but little is known about the contribution of a primary deficiency in liver regeneration. We developed a mouse model to assess the regenerative capacity of a critically small liver remnant.

Methods: Extended (86%) hepatectomy (eHx) was modified to minimize collateral damage; effects were compared with those of standard (68%) partial hepatectomy (pHx) in mice. Markers of liver integrity and survival were evaluated after resection. Liver regeneration was assessed by weight gain, proliferative activity (analyses of Ki67, proliferating cell nuclear antigen, phosphorylated histone 3, mitosis, and ploidy), and regeneration-associated molecules. Knockout mice were used to study the role of p21.

Results: Compared with pHx, survival of mice was reduced after eHx, and associated with cholestasis and impaired liver function. However, no significant differences in hepatocyte death,

sinusoidal injury, oxidative stress, or energy depletion were observed between mice after eHx or pHx. No defect in the initiation of hepatocyte proliferation was apparent. However, restoration of liver mass was delayed after eHx and associated with inadequate induction of Foxm1b and a p21-dependent delay in cell-cycle progression. In $p21^{-/-}$ mice, the cell cycle was restored, the gain in liver weight was accelerated, and survival improved after eHx.

Conclusions: Significant parenchymal injury is not required for liver failure to develop after extended hepatectomy. Rather, liver dysfunction after eHx results from a transient, p21-dependent block before hepatocyte division. Therefore, a deficiency in cell-cycle progression causes liver failure after extended hepatectomy and can be overcome by inhibition of p21.

Abstract taken from (143).

6.4. Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet

Ehses JA, Meier DT, Wueest S, Rytka J, Boller S, Wielinga PY, Schraenen A, Lemaire K, Debray S, Van Lommel L, Pospisilik JA, Tschopp O, Schultze SM, Malipiero U, Esterbauer H, Ellingsgaard H, Rütti S, Schuit FC, Lutz TA, Böni-Schnetzler M, Konrad M, Donath MY.

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Abstract

Aims/hypothesis: Inflammation contributes to both insulin resistance and pancreatic beta cell failure in human type 2 diabetes. Toll-like receptors (TLRs) are highly conserved pattern recognition receptors that coordinate the innate inflammatory response to numerous substances, including NEFAs. Here we investigated a potential contribution of TLR2 to the metabolic dysregulation induced by high-fat diet (HFD) feeding in mice.

Methods: Male and female littermate *Tlr2* $^{+/+}$ and *Tlr2* $^{-/-}$ mice were analysed with respect to glucose tolerance, insulin sensitivity, insulin secretion and energy metabolism on chow and HFD. Adipose, liver, muscle and islet pathology and inflammation were examined using molecular approaches. Macrophages and dendritic immune cells, in addition to pancreatic islets were investigated in vitro with respect to NEFA-induced cytokine production.

Results: While not showing any differences in glucose homeostasis on chow diet, both male and female *Tlr2* $^{-/-}$ mice were protected from the adverse effects of HFD compared with *Tlr2* $^{+/+}$

littermate controls. Female *Tlr2* $^{-/-}$ mice showed pronounced improvements in glucose tolerance, insulin sensitivity, and insulin secretion following 20 weeks of HFD feeding. These effects were associated with an increased capacity of *Tlr2* $^{-/-}$ mice to preferentially burn fat, combined with reduced tissue inflammation. Bone-marrow-derived dendritic cells and pancreatic islets from *Tlr2* $^{-/-}$ mice did not increase IL-1β expression in response to a NEFA mixture, whereas *Tlr2* $^{+/+}$ control tissues did.

Conclusion/interpretation: These data suggest that TLR2 is a molecular link between increased dietary lipid intake and the regulation of glucose homeostasis, via regulation of energy substrate utilisation and tissue inflammation.

Abstract taken from (56).

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8. Curriculum vitae

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List of publications

- **Schultze SM**, Hynx D, Geier A, Niessen M, Spinas GA, Hemmings BA, Tschopp O. *AKT2/PKBβ activation in skeletal muscle regulates hepatic lipid content in Pten-haplodeficient mice.* (manuscript submitted)
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Attended Conferences

- TOR, PI3K and Akt - 20 years on; Basel, 2011
  Poster presentation
- Internal FMI annual meeting; 2009-2012
  Poster presentation
- Stress, Signalling and Cancer; Madrid, 2008
  Poster presentation